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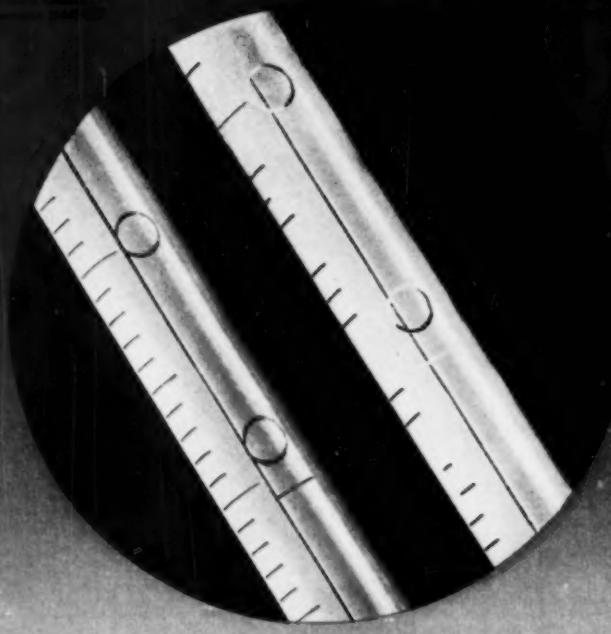

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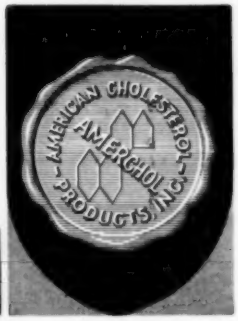

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Chemotherapy of Experimental Relapsing Fever in Mice with Antibiotics and Synthetic Compounds*

By PAUL E. THOMPSON, D. F. WALKER, and MARY C. DUNN

In a search for new antispirochetal agents, four antibiotics and 206 synthetic compounds were examined by testing their ability to suppress the spirochetemia in standardized relapsing fever infections in mice. Subtilin, methylated subtilin, bacitracin, and Melarsen oxide were found to be very effective.

THIS PAPER PRESENTS results from the screening of various substances for action against relapsing fever spirochetes. While interest in the chemotherapy of relapsing fever provided encouragement for the study, the premise that relapsing fever infections in mice may serve as a convenient test for general antispirochetal action served as an additional impetus for the work and the reporting of these results.

MATERIALS AND METHODS

Identity of the strain of *Borrelia novyi* along with information on strain maintenance, the nature of experimental infections, and procedures for initiating and studying infections during chemotherapeutic tests have been described elsewhere (1).

The present data were collected under the following relatively standardized conditions. The mice, with few exceptions, were females weighing 15–20 Gm., purchased from Harlan Farms. The infecting inoculum was procured by drawing blood by cardiac puncture from donor mice twenty-four to forty-eight hours after infection. Infections were induced by the intraperitoneal injection of 1×10^{10} spirochetes/Kg. of body weight. Drugs were given intraperitoneally in three doses during a one and one-half day period, individual doses at approximately four, twenty-four, and thirty hours after infection. Evidence of the suitability of infecting and treating by the same route had been provided by observing that the number of spirochetes in the circulating blood was similar at three hours after infection by the intraperitoneal and intravenous routes. Drugs were given as aqueous solutions or as suspensions in 10% acacia in a volume of 10–20 ml./Kg. of body weight and within a *p*H range of 5.5–8.5. Dosages are expressed in terms of the free base. The effects of therapy were determined primarily on the basis of suppression of the number of spirochetes in the circulating blood at forty-eight hours after infection and to a lesser extent on the basis of protection afforded animals against death during seven days after infection. The level of spirochetemia was measured by counting the number of spirochetes/10,000 erythrocytes in stained smears of the peripheral blood. In each experiment a group of mice was infected from a common pool of spirochetes and was divided into subgroups. One subgroup of 10 was used as infection controls, one subgroup of 5 was treated with a reference drug and the remaining subgroups of 5 were treated with new drugs. Crystalline sodium penicillin G (potency, 1667 international units/ml.) and Mapharsen[1] (2-amino-4-arsenosophenol hydrochloride) were used as reference drugs for antibiotics and synthetic compounds, respectively. Many published reports and extensive usage have shown that both of these compounds are potent antispirochetal drugs. The antispirochetal action of a drug, determined from its effect on the spirochetemia, is expressed in terms of the reference drug equivalent, i. e., the ratio of the effective dose of the reference drug to that of the test

* Received June 18, 1953, from the Research Laboratories, Parke, Davis and Company, Detroit, Mich.

[1] Parke, Davis and Company trade mark for oxophenarsine hydrochloride.

TABLE I.—DATA FROM SCREENING TESTS ON THE NATURE OF UNTREATED INFECTIONS AND THE PERFORMANCE OF THE REFERENCE DRUGS

Identity	Mice: Total No.	Mice: No./ Group	Mice: No. of Groups	Drug, Mg./Kg./ Day[a]	Spirochetemia Means[b]: Mean	Spirochetemia Means[b]: Range	Spirochetemia Suppression Means, %[c]: Mean	Spirochetemia Suppression Means, %[c]: Range	Host Mortality[d] Means, %: Mean	Host Mortality[d] Means, %: Range
Untreated	490	10	49	...	8,211	5,070–11,510	..	...	77	0–100
Penicillin G	35	5	7	12.0	1,844	1,020–2,590	71	51–86	20	0–40
Mapharsen	145	5	29	3.2	3,632	1,560–6,760	58	16–81	52	0–100

[a] Drugs were given intraperitoneally for one and one-half days (3 doses; 4, 24, and 30 hours after infection).
[b] Spirochetes per 10,000 erythrocytes at approximately 48 hours after infection with 1×10^{10} spirochetes/Kg. of body weight.
[c] Relative to a parallel control group of 10 untreated mice.
[d] Through 7 days after infection.

compound. These equivalents are based on 5.7 mg./Kg./day for penicillin G, the estimated ED_{50} (1) and on 3.2 mg./Kg./day for Mapharsen, the estimated ED_{50} (*infra*). Reference drug equivalents for active substances probably are accurate within a factor of 2, but those for inactive drugs frequently may not be accurate within a factor of less than 4. Most substances were tested without prior information on toxicity and were given to 3 groups of animals in doses of 800, 200, and 50 mg./Kg./day, respectively. Unless activity was encountered, further testing was not done. The largest amount of a drug actually tested can be determined by dividing the assigned equivalent into the reference drug dose. Thus a Mapharsen equivalent of <0.064, <0.016, or <0.004 indicates the compound was tolerated but inactive in doses of 50, 200, or 800 mg./Kg./day for one and one-half days, respectively. The letter "*T*" after an equivalent means that the drug was fatally toxic for at least 2 of 5 animals at four times the dose level used in assigning its reference drug equivalent. Thus, drug equivalents give a rough estimate of antispirochetal activity and some idea of host toxicity under the conditions of this study.

RESULTS

Untreated Infections and Infections Treated with the Reference Drugs.—The general host-parasite relationships in untreated acute *B. novyi* infections of the mouse, under conditions of this study, have been described (1, 2). The infection rate in several hundred untreated mice has been 100%. Data on the level of blood infection and host mortality of untreated mice (infection controls) and of mice treated with certain levels of penicillin G or of Mapharsen (drug controls) are summarized in Table I. These data were compiled from many screening tests.

The suppressive action of penicillin G on the spirochetemia is characterized by a linear relationship between dose and effect and the ED_{50} is approximately 5.7 mg./Kg./day (1). Since this value was observed under the conditions of and concurrently with the present study it seems more appropriate to use it in assigning penicillin G equivalents than the dose most frequently used in screening tests, 12.0 mg./Kg./day, which on the average conferred 71% suppression.

Preliminary observations indicated the suppressive action of Mapharsen on the spirochetemia to be proportional to the amount of drug. Mapharsen equivalents are based on 3.2 mg./Kg./day since this dose of Mapharsen was used most frequently and probably provides the best available estimate of its action, namely, on the average 58% suppression of the spirochetemia.

The Antispirochetal Action of Various Antibiotics.—The performance of Chloromycetin[2] in comparison with that of penicillin G against relapsing fever spirochetes has been described in an earlier report (1), representing a part of this general study. The penicillin G equivalent for the suppressive action of Chloromycetin was estimated to be 0.37. Although comparison of the protective action of these drugs against host mortality was complicated by dissimilarity in their dose-protection relationships, Chloromycetin more dependably protected a high percentage of mice against a uniformly lethal inoculum than did penicillin G at the same

[2] Parke, Davis and Company trade mark for chloramphenicol.

TABLE II.—EFFECTS OF BACITRACIN[e] ON RELAPSING FEVER INFECTIONS IN MICE

Drug Dose, Mg./Kg./Day[a]	Spirochetemia Means[b]	Suppression of Spirochetemia, %[c]	Host Mortality, Deaths/Total[d]	Expt. No.
200	0	100	0/5	27
50	2	>99	0/5	27
25	24	>99	1/5	29
12.5	80, 252	99, 96	0/5, 1/5	27, 29
6.25	2,318, 3,660	65, 45	0/5, 0/5	29, 32
3.13	5,440, 5,680	11, 7	0/5, 0/5	27, 32
1.56	5,240	14	0/5	32
0	6,100		5/10	27
0	6,580		4/10	29
0	6,125		1/10	32

[a–d] Same as in Table I.
[e] Lot No. 4801068.

TABLE III.—EFFECTS OF PENICILLIN G AND BACITRACIN, ALONE AND IN COMBINATION, ON RELAPSING FEVER INFECTIONS IN MICE

Group No.	Penicillin G		Bacitracin		Penicillin G plus Bacitracin (Sum of ED_{50}'s)[d]	Suppression of Spirochetemia, %[b]	Host Mortality, Deaths/Total[c]
	Mg./Kg./Day[a]	Fraction of ED_{50}[d]	Mg./Kg./Day[a]	Fraction of ED_{50}[d]			
1	9.0	0.87	0	...	...	30	1/5
2	12.0	1.15	0	...	...	72	1/5
3	14.4	1.38	0	...	...	87[e]	1/5
4	0	...	4.4	0.81	...	28	0/5
5	0	...	6.6	1.22	...	75	0/5
6	0	...	8.6	1.59	...	91	0/5
7	4.5	0.43	2.2	0.41	0.84	86	0/5
8	6.0	0.58	3.3	0.61	1.19	97[e]	1/5
9	7.2	0.69	4.3	0.80	1.49	98	0/5
10	0	...	0	(Untreated controls)		(9274 Spirochetes per 10,000 R. B. C.)	8/10

[a–c] Same as in Table I.
[d] ED_{50}'s, estimated on log-probit scales from this experiment, were 10.4 mg./Kg./day for penicillin G and 5.4 mg./Kg./day for bacitracin.
[e] One animal died before blood smears were made and is not included.

dose level. The superiority of Chloromycetin in this respect may be explained by its more rapid action, as observed during *in vitro* tests, and by its greater diffusibility in the tissues. Evidence of broad antispirochetal action by Chloromycetin is provided by reports on its therapeutic effects in human syphilis (3, 4), rabbit syphilis (5), yaws (6), relapsing fever of man and animals (7), and avian spirochetosis (8).

The effects of bacitracin[3] are presented in Tables II and III. Perusal of these data indicates bacitracin has a penicillin equivalent of approximately one to two. Evidence that treatment with bacitracin confers substantial reduction in early host mortality also is provided in these tabulations. Bacitracin also has been found to be active in rabbit syphilis (3).

A strong suggestion that penicillin and bacitracin act synergistically against relapsing fever spirochetes is provided by the results from a single experiment, see Table III. This suggestion, which is in accord with an observation of synergistic action by these drugs in rabbit syphilis (9), is most clearly apparent from a comparison of the amount of suppression of the spirochetemia associated with fractions of the ED_{50}'s of the drugs. Estimates of the ED_{50}'s are, appropriately, restricted to data from this experiment and differ somewhat from estimates based on larger amounts of data (*supra*). The degree of suppression associated with the sums of the ED_{50} fractions of the two drugs (groups 7, 8, and 9) exceeded in each instance the degree of suppression produced by equivalent ED_{50} fractions of each drug alone (groups 1 and 4, 2 and 5, 3 and 6, respectively).

Three subtilin preparations were used in studying the antispirochetal action of this antibiotic. Data on two of these, subtilin Lot 242[4] and methylated subtilin,[4] are presented in Table IV. The penicillin equivalents of these preparations were approximately 7.2 and 2.4, respectively. Data on the third subtilin preparation, Lot 32-B,[5] which for the sake of brevity are not tabulated, indicated the penicillin equivalent was approximately 1.3. Concurrent microbiological assays with *Staphylococcus aureus* of these subtilin preparations in comparison to a fourth lot of subtilin, used as a standard, demon-

[3] Lot 480106S, labeled potency 44 units/mg., kindly supplied by the Commercial Solvents Corporation.

[4] Subtilin Lot 242 and methylated subtilin Lot L2003 were kindly supplied by Dr. J. C. Lewis of the Western Regional Research Laboratories, U. S. Department of Agriculture.
[5] Subtilin Lot 32-B, kindly supplied by the Wallerstein Company.

TABLE IV.—THE EFFECTS OF SUBTILIN LOT 242 AND METHYLATED SUBTILIN ON RELAPSING FEVER INFECTIONS IN MICE

Drug Dose, Mg./Kg./Day[a]	Subtilin			Methylated Subtilin		
	Spirochetemia Suppression, %[b]	Host Mortality, Deaths/Total[c]	Expt. No.	Spirochetemia Suppression, %[b]	Host Mortality, Deaths/Total[c]	Expt. No.
25	100	0/5	27	100	0/5	27
12.5	100	0/5	27	>99	0/5	27
6.25	100	0/5	27	>99	0/5	27
3.13	100, 100	0/5, 0/5	27, 29	72, 47	0/5, 2/5	27, 29
1.56	98	0/5	29	30, 11	0/5, 2/5	29, 31
1.0	79	0/5	33	...	...	
0.8	25	1/5	33	...	...	
0.78	76, 52	0/5, 0/5	29, 31	27[d]	2/5	31
0.39	0	1/5	31	0	2/5	31
	Spirochetes per 10,000 R. B. C.					
Untreated Controls	6,100	5/10	27			
	6,580	4/10	29			
	5,454	1/10	31			
	5,267	1/10	33			

[a–c] Same as in Table I.
[d] One animal died before blood smears were taken and is not represented in these data.

TABLE V.—SYNTHETIC COMPOUNDS TESTED AS ANTISPIROCHETAL AGENTS

Name of Compound	Mapharsen Equivalent, *M*
I. Aliphatic Compounds	
Chloroformylmethionic acid, dipotassium salt*, [f]	<0.004
1-Carbamylcyclopropanecarboxylic acid, ethyl ester*, [e]	<0.004
γ-Carboxyethyl-γ-nitropimelic acid†	<0.004
3-Hexene-2,5-dione*, [a]	<0.025*T*
N,N-Bis(2-hydroxyethyl)glycine*, [a]	<0.004
Oxalic acid, dihydrazide†, [e]	<0.004
Oxalyldicarbamic acid, diethyl ester†, [e]	<0.004
β,β′-Sulfonyldipropionic acid†, [i]	<0.004
Xanthic acid, ester w. hydracrylic acid†, [i]	<0.016
II. Aromatic Compounds	
A. Alcohols	
1,3-Bis(4-aminophenoxy)-2-propanol†	<0.004
α-[2-(Hydroxyethylamino)methyl]benzyl alcohol*, [a]	<0.004
4-Iodobenzyl alcohol*, [e]	<0.016
2,2′-(*m*-Phenylenedioxy)diethanol†, [a]	<0.004
β-Phenyl-1-piperidine ethanol, hydrochloride*	<0.016*T*
B. Carboxylic Acids	
Benzilic acid†	<0.004
γ-Benzoylbutyric acid†, [e]	<0.004
β-Benzoylpropionic acid†, [e]	<0.004
γ-Benzoylvaleric acid†, [e]	<0.008
10-(4-Butyl-*x*-carboxy-2-cyclohexen-1-yl)-9-decenoic acid†, [e]	<0.016*T*
Dibromotyrosine†	<0.004
3,4-Dihydroxyphenylacetic acid*, [e]	<0.004
Homoveratric acid*, [e]	<0.004
p-Dimethylsulfamylbenzoic acid†, [e]	<0.004
3,4,5,6-Tetrachloro-2-(2,5-dichlorobenzoyl)benzoic acid†, [e]	<0.064*T*
N-(5-Hydroxy-1-naphthyl)glycine, hydrochloride†, [e]	<0.004
4-Methylhydrocinnamic acid†, [e]	<0.004
4-(N-Methylacetamido)benzoic acid*, [e]	<0.004
β-(*p*-Toluyl)propionic acid†, [e]	<0.016
2-Phenoxybenzoic acid†, [f]	<0.064*T*
α-Phenylbutyric acid†, [e]	<0.004
β-(Phenylmercapto)propionic acid†,	<0.064*T*
Protocatechuic acid*, [e]	<0.004
Pyromellitic acid*, [e]	<0.004
2,3,5-Triiodobenzoic acid†, [e]	<0.016*T*
β-Resorcylic acid, ($^1/_2H_2O$)*	<0.004
N-(2,4,6-Triiodophenoxyacetyl)glycine†, [e]	<0.016*T*
C. Esters	
Benzhydrylthionocarbamic acid, β-diethylaminoethyl ester, hydrochloride†, [j]	<0.064*T*
N-Hexyl-α-phenylglycine, ethyl ester, hydrochloride†	<0.016*T*
2-(4′-Aminophenoxy)benzoic acid, β-diethylaminoethyl ester, hydrochloride*, [j]	<0.004
α-(4-Iodophenyl)-*n*-butyric acid, 3-hydroxypropyl ester†, [e]	<0.016*T*
1-(Mercaptoacetyl)-3-phenylurea, S-acetate ester†, [e]	<0.004
β-Phenyl-1-piperidine ethanol, benzoate ester, hydrochloride†	<0.004
Methyl 4-dimethylaminobenzoic acid, methyl ester†, [e]	<0.004
D. Amides	
N-Butyl-N-(β-hydroxyethyl)benzenesulfonamide, acetate ester†, [g]	<0.004
p-Acetylsulfamylbenzoic acid, (H_2O)†	<0.004
p-(N,N-Dibenzylglycyl)acetanilide†, [i]	<0.016*T*
4-(4-Dimethylamino-2-tolylazo)acetanilide*, [g]	<0.004
N-Ethyl-N-(β-hydroxyethyl)benzenesulfonamide, acetate ester†, [g]	<0.004
4-Methoxybenzamide†, [e]	<0.004
4-(4-Morpholinylacetyl)acetanilide†, [i]	<0.004
α-(1-Naphthylamino)acetamide†, [e]	<0.004
Tuio-2-cyclohexene-1-carboxamide†, [a]	<0.016*T*
E. Amines	
4-Aminoacetophenone†, [e]	<0.004
4-Amino-α-toluenephosphonic acid†, [k]	<0.004
5-Amino-2′,6′-diiodo-2,4′-iminodibenzenesulfonic acid*, [e]	<0.004
N-(*p*-Aminophenyl)anthranilic acid†, [e]	<0.004
6-Amino-*m*-toluenesulfonic acid*, [e]	<0.064*T*
N,N-Bis[2-(*p*-chlorophenoxy)ethyl]cyclohexylamine†, [c]	<0.004
5,5′-(Dibromovinylidene)di-γ-anisidine†, [q]	<0.004
5,5′-(Dichlorovinylidene)di-γ-anisidine†, [q]	<0.016*T*
5,5′-(Dichlorovinylidene)di-γ-toluidine†, [q]	<0.005
N,N-Dimethylmetanilic acid, sodium salt*, [c]	<0.004
2,4,6-Triaminophenol, trihydrochloride*, [e]	<2.0*T*
4-Aminopyrocatechol, hydrobromide†, [e]	<0.016*T*
F. Arsenicals	
2,4-Diamino-6-(4-arsenosoanilino)-*s*-triazine, (H_2O), (Melarsen oxide)‡	2.0
2-Amino-4-arsenosophenol, hydrochloride, (Mapharsen)*	1.0
(*p*-Carbamylphenylarsenylenedithio)diacetic acid, (arsenamide)*	0.15
G. Miscellaneous	
Germanin*	<0.03
[9,9′-Bifluorene]-2,2-dicarboxamidine, dihydrochloride*	<0.13*T*
2-*t*-Butyl-1,4-naphthoquinone†, [b]	<0.016*T*
1,1-Dichloro-2,2-bis(4-methoxy-3-nitrophenyl)ethylene†, [q]	<0.004
4,4′-Dihydroxybenzophenone†	<0.004
(2,4-Disulfophenoxy)acetic acid, *p*-toluidine salt†	<0.064*T*
4-(2-Nitrovinyl)guaiacol†	<0.064*T*
3,4-Dihydroxyacetophenone*, [e]	0.004
N-(β-Hydroxyethyl)-4,5,6,7-tetraiodophthalimide†, [e]	<0.004
2-Hydroxy-α-methyl-1-naphthaleneacetonitrile†	<0.016*T*
3-Phenoxy-1-propanesulfonic acid, sodium salt*, [e]	<0.004
2′,5′-Dihydro-2′,5′-dioxo-4-biphenylsulfonic acid, sodium salt†, [e]	<0.016*T*
N-(?)-Trifluoromethylsulfanilic acid†, [e]	<0.004
1,1,1-Trichloro-2,2-bis(4-methoxy-3-nitrophenyl)ethane†, [q]	<0.004
1-(4-Hydroxy-3-methoxyphenyl)-1,2-propanedione†, [e]	<0.004
4-Hydroxyacetophenone†	<0.004
4-Hydroxybenzophenone†	<0.004
4-Hydroxypropiophenone†, [e]	<0.004
III. Heterocyclic Compounds	
A. Pyridines	
(3-Aminophenyl)-4-pyridyl ketone†, [l]	<0.004
N-Decylnicotinamide†,	<0.004
3-Cyano-2,6-dihydroxyisonicotinic acid, ethyl ester*, [e]	<0.004
3,5-Diiodo-4(1)-pyridone†, [e]	<0.004
1,1′-Dimethylnicotinium di-*p*-toluenesulfonate*	<0.008
4,4′-Dithiobis[1-methyl-2-(3-pyridyl)pyrrole]†, [d]	<0.004
5,5′-Methylenebis-(2-dimethylaminopyridine)†, [l]	<0.004
N-[3-(3-Pyridylcarbonyl)propyl]benzamide†	<0.004
N-2-Pyridylnicotinamide†, [d]	<0.016*T*
1-Phenyl-3-(2-pyridyl)urea†	<0.004
B. Piperidines	
3-(4-Aminobutyl)piperidine, dihydrochloride*	<0.026*T*
α-Phenyl-1-piperidineacetic acid, isopropyl ester, hydrochloride†	<0.004
α-Piperidineacetic acid, methyl ester, hydrochloride†	<0.004
α-Phenylpiperidineacetic acid, propyl ester, hydroiodide†	<0.064*T*
3-(2-Pyrrolidyl)piperidine, dihydrochloride*	<0.006
C. Pyrimidines	
2-Amino-4-(N-ethylanilino)pyrimidine†	<0.064*T*
2-(α-Mercaptoacetamido)pyrimidine, acetate ester†, [e]	<0.004
G. Imidazoles	

	N-Ethyl-N-(β-hydroxyethyl)benzenesulfonamide, acetate ester†, [g]	<0.004
	4-Methoxybenzamide†, [c]	<0.004
	4-(4-Morpholinylacetyl)acetanilide†, [j]	<0.004
	2-(α-Mercaptoacetamido)pyrimidine, acetate ester†, [e]	<0.004
	5-(4-Nitrophenylmercapto)uracil†	<0.004
	1-Phenyl-3-(2-pyrimidyl)urea†	<0.004
D.	Triazines	
	2-Amino-4-(α-mercaptoacetamido)-6-methyl-*s*-triazine, acetate ester†, [e]	<0.016*T*
	2-(4-Amino-6-anilino-2-*s*-triazinylamino)ethanol†	<0.064*T*
	N^2-Allyl-N^4-phenylmelamine†	<0.004
	4-(4-Amino-6-dimethylamino-2-*s*-triazinylamino)salicyclic acid†	<0.016
	2-Amino-4-(4-chloroanilino)-6-(4-morpholinyl)-*s*-triazine†	<0.004
	2-Amino-4-dimethylamino-6-ethoxy-*s*-triazine†	<0.016*T*
	2-Amino-4-butoxy-6-butylamino-*s*-triazine†	<0.016*T*
	2-Amino-4-butoxy-6-diallylamino-*s*-triazine†	<0.004
	2-Amino-4-butoxy-6 (2-methallylamino)-*s*-triazine†	<0.004
	2-Amino-4-[N-(2-chloroethyl)anilino]-6-methoxy-*s*-triazine†	<0.016*T*
	2-[4-Amino-6-(*p*-chloroanilino)-2-*s*-triazinylamino]ethanol†	<0.016*T*
	2-[4-Amino-6-methoxy-2-*s*-triazinyl)ethylamino]ethanol†	<0.004
	2-[N-(4-amino-6-ethoxy-2-*s*-triazinyl)anilino]ethanol†	<0.004
	(4-Allylamino-6-amino-2-*s*-triazinyloxy)acetic acid†	<0.128*T*
	2-Anilino-4,6-di-4-morpholinyl-*s*-triazine†	<0.016*T*
	4,4′-Bis(4,6-dihydroxy-2-*s*-triazinylamino)-2,2′-stilbenedisulfonic acid, disodium salt*	0.01
	4,4′-Bis(4-amino-6-hydroxy-2-*s*-triazinylamino)-2,2′-stilbenedisulfonic acid, disodium salt†, [e]	<0.004
	N^4-(*m*-chlorophenyl)melamine†	<0.064*T*
	N-Benzyl-N-phenylmelamine†	<0.016*T*
	N^2-*p*-Chlorophenyl-N^4,N^6-dimethylmelamine†	<0.064*T*
	N^2,N^2-Diallyl-N^4-(*p*-chlorophenyl)melamine†	<0.004
	p-(4,6-Diamino-2-*s*-triazinylamino)phenol, hydrochloride†	<0.016
	N-(3,4-Dichlorophenyl)melamine†	<0.016*T*
	2,4-Diamino-6-methoxy-*s*-triazine†	<0.004
	2,4-Diamino-6-propoxy-*s*-triazine†	<0.064*T*
	2,4-Diamino-6-(2-ethoxyethoxy)-*s*-triazine†	<0.004
	2,4-Diamino-6-[2-(4-morpholinyl)ethoxy]-*s*-triazine†	<0.016
	2,4-Diamino-6-(3-diethylaminopropoxy)-*s*-triazine†	<0.016
	2,4-Diamino-6-(4-morpholinyl)-*s*-triazine†	<0.016*T*
	N^2,N^4-Diethyl-N^6-phenylmelamine†	<0.016
	N^2,N^4-Diallyl-N^6-(*p*-chlorophenyl)melamine†	<0.016*T*
	2,4-Diamino-6-phenyl-*s*-triazine†, [c]	<0.016*T*
	2,4-Diamino-6-benzyl-*s*-triazine†, [c]	<0.004
	(4,6-Diamino-2-*s*-triazinylmercapto)acetic acid, sodium salt†, [c]	<0.004
	N-Methyl-N-phenylmelamine†	<0.016
	N-(*p*-Nitrophenyl)malamine†	<0.064*T*
	2,2′-(Oxydiethylenedithio)bis(4,6-diamino-*s*-triazine)†, [e]	<0.016*T*
E.	Quinolines	
	(8-Hydroxyquinolyl)methylketone†	<0.064*T*
	2-(4,5-Dimethyl-2-thiazolylmercapto)quinoline†, [h]	<0.004
	2-(*o*-Hydroxyphenyl)benzo[H]quinoline-4-carboxylic acid*, [e]	<0.004
	2-(*o*-Hydroxyphenyl)-8-sulfobenzo[H]quinoline-4-carboxylic acid, disodium salt*, [e]	<0.004
	2-Phenylmercaptoquinoline†	<0.004
F.	Piperazines	
	1,4-Bis(3-nitro-4-tolylsulfonyl)piperazine†, [m]	<0.004
	1,4-Bis(phenylsulfonyl)piperazine†, [m]	<0.018
	1,4-Bis(*o*-tolylsulfonyl)piperazine†, [m]	<0.004
	1-(2-Ethoxy-4-quinolylcarbonyl)-4-phenylpiperazine†, [m]	<0.004
	Piperazine, bis benzenesulfonate salt*, [m]	<0.004
	Piperazine, dicaprylate salt*, [m]	<0.004
	Piperazine, di-2-hydroxycinchoninic acid salt*, [m]	<0.004
	1-Phenylpiperazine, hydrochloride*, [m]	<0.064*T*
G.	Imidazoles	
	5-Dibenzylamino-1-methyl-4-nitroimidazole†, [i]	<0.004
	Phenyl-(4-phenyl-2-imidazolyl)ketone†, [a]	<0.004
	N-(6-Benzimidazolyl)-*p*-toluenesulfonamide†, [e]	<0.064*T*
	2-Benzimidazolepropionic acid†, [e]	<0.004
	5-Dibenzylamino-1-methyl-4-nitroimidazole†, [j]	<0.004
H.	Quinazolines	
	6-Amino-4(3H)-quinazolone†, [p]	<0.064*T*
	6-Chloro-4-ethoxyquinazoline†, [p]	<0.016*T*
	4-Chloro-2-phenylquinazoline†, [p]	<0.016
	4,6-Dichloroquinazoline†, [p]	<0.064*T*
	4-(4-Diethylamino-1-methylbutylamino)-6-methoxyquinazoline*, [p]	<0.064*T*
	4-(4-Diethylamino-1-methylbutylamino)-2-phenylquinazoline, diphosphate, (H_2O)*, [p]	<0.016*T*
	6-Nitro-4(3H)-quinazolone†, [p]	<0.016*T*
	2-Phenyl-4(3H)-quinazolone†, [p]	<0.005
	4-(3H)quinazolone†, [p]	<0.004
I.	Miscellaneous Heterocycles	
	Quinacrine, dihydrichloride, ($2H_2O$)*	<0.016*T*
	Alizarin geranol*, [e]	<0.004
	3-(2-Aminoethyl)-2-(3-pyridyl)indole, dihydrochloride†	<0.016*T*
	5-Amino-1,2,3,4-thiatriazole*, [e]	<0.016
	2-Amino-3-ethyl-6-methylbenzothiazolium ethyl sulfate†, [e]	<0.064*T*
	AR-(α-Mercaptoacetamido)-2-benzoxazolethiol, α5-acetate ester†, [e]	<0.004
	α,α-Bis[4-Dimethylaminophenyl]-2-benzothiazole methanol†, [k]	<0.004
	2-Benzothiazolylphenyl ketone†, [k]	<0.004
	1,3-Bis(hydroxymethyl)-2-(3H)benzamidazolethione†	<0.064*T*
	2-Benzyl-9H-fluoreno[3,2]oxazole†, [n]	<0.004
	α-(*p*-Chlorophenyl)-α-methyl-2-benzothiazolemethanol†, [k]	<0.004
	3,5-Dimethyl-3,4-pyrroledicarboxylic acid, 4-ethyl ester*, [e]	<0.016*T*
	2,4(1,3)-Quinazolinedione†, [e]	<0.064*T*
	α,α-Diphenyl-2-benzothiazolemethanol†, [k]	<0.004
	α-(*p*-Dimethylaminophenyl)-2-benzothiazolemethanol†, [k]	<0.004
	4-Dibenzothiophenecarboxamide†, [k]	<0.016*T*
	4-Dibenzothiophenecarboxamide-5-dioxide†, [k]	<0.016*T*
	2,4-Diaminothiazole, hydrochloride*, [a]	<0.004
	Guanazole, dihydrochloride*, [e]	<0.016*T*
	7-Isoquinolinol†, [e]	<0.016*T*
	3-[1-(2-Hydroxyethylimino)ethyl]-6-methyl-1,2H-pyran-2,4(3H)-dione*, [a]	<0.016*T*
	γ-Hydroxy-β-methyl-α-hydromucic acid, γ-lactone*	<0.004
	3-(Hydroxymethyl)-2-(3)-benzothiazolethione†	<0.064
	2,2′-Methylenedithio bis-2-thiazoline†, [a]	<0.016*T*
	2-Imino-3-benzothiazolineacetic acid†, [e]	<0.016*T*
	Mercaptoacetic acid, 2-(1,2,3,4-tetrazol-5-YL)hydrazide, 5-acetate ester†, [e]	<0.016*T*
	α-Methyl-α-*p*-tolyl-2-benzothiazolemethanol†, [k]	<0.016
	3-Methylrhodanine†, [a]	<0.016*T*
	6-Nitrobenzazimidol†, [e]	<0.004*T*
	α-Methyl-α-phenyl-2-benzothiazolemethanol†, [k]	<0.004
	α-Propyl-2-benzothiazolemethanol†, [k]	<0.016*T*
	α-Phenyl-2-benzothiazolemethanol†,	<0.004
	5-Oxo-5-N-thiazolo[3,2-A]pyrimidine-6-carboxylic acid†	<0.016*T*
	4,6,6-Trimethyl-1,3,6-thiazine-2-thiol†, [l]	<0.004
	2,3,5,6-Tetramethylpyrazine (H_2O)†	<0.004
	N-(1,2,3,4-Tetrazol-5-yl)-*p*-toluenesulfonamide†, [e]	<0.016*T*
	(3-Hydroxy-2-quinoxalylcarbonyl)urea†, [e]	<0.016*T*
	2,7-Diacetamidoxanthone†,	<0.016*T*
	α-Acetyl-9-xantheneacetic acid, ethyl ester†, [l]	<0.004

State of drug and menstruum: * solution in water; † suspension in 10% acacia; ‡ solution in 5% propylene glycol.

T = Toxic as defined in section on Materials and Methods.

Key to sources of compounds from outside suppliers: [a] Carbide and Carbon Chemicals Company; [b] Dr. H. W. Davis, University of South Carolina; [c] The Dow Chemical Company; [d] Eastern Regional Research Laboratories; [e] Eastman Kodak Company; [f] General Chemical Division; [g] General Mills, Inc.; [h] Dr. Henry Gilman, Iowa State University; [i] The B. F Goodrich Company; [j] Dr. C. S. Hamilton, University of Nebraska; [k] Monsanto Chemical Company; Dr. H. S. Mosher, Stanford University; [m] Dr. C. B. Pollard, University of Florida; [n] Dr. F. E. Ray, University of Florida; [o] Rohm and Haas Company; [p] Dr. M. L. Sherrill, Moint Holyoke College; [q] Dr. D. A. Shirley, The Tulane University of Louisiana.

strated the following antibacterial potencies, expressed as per cent of the standard: subtilin Lot 242, 208; methylated subtilin, 96; subtilin Lot 32-B, 42.[6] Thus, parallelism was observed between the antispirochetal and antibacterial activities of the three preparations. Antibacterial assays showed the sample of methylated subtilin deteriorated during storage from an estimated potency of 297 to 96% of the standard. Presumably, if it had been tested in the present work before appreciable deterioration had occurred its antispirochetal activity would have been of the order of that observed with subtilin Lot 242, a relatively stable and relatively pure preparation. All three subtilin preparations conferred considerable portection to the mice against death from acute spirochetosis.

The high activity of subtilin against relapsing fever spirochetes is in contrast to its effects in rabbit syphilis (10). In the latter it had not more than slight effect, and while it was active *in vitro* against the Reiter strain of *Treponema pallidum*, the minimal effective concentrations were of the order of 75 to 100 times those of penicillin.

Streptomycin (data not tabulated) conferred suppression of the spirochetemia proportional to the amount administered. However, the ED_{50} appeared to be 200 mg./Kg./day, giving a penicillin equivalent of approximately 0.03. Evidence of action of streptomycin in relapsing fever has been observed by others [see Schuhardt (11) for a review of the literature].

The Antispirochetal Action of Various Synthetic Compounds.—Screening results on 206 compounds are presented in Table V. A wide variety of chemical types were tested in a search for new active agents exclusive of the organo-metallic compounds. Only two such compounds were found to be active. One of these is the antitrypanosome drug, germanin or Bayer 205, which had a Mapharsen equivalent of 0.03. The other compound is 4,4′-bis(4,6-dihydroxy-2-*s*-triazinylamino)-2,2′-stilbenedisulfonic acid, disodium salt, which had a Mapharsen equivalent of 0.01. Neither of these compounds appears to be sufficiently active to warrant further consideration in the treatment of relapsing fever. Testing of various compounds related to the above triazine failed to reveal other active agents.

In addition to Mapharsen, two arsenicals were tested and found to be active. One of these is 2,4-diamino-6-(4-arsenosoanilino)-*s*-triazine hydrate (Melarsen[7] oxide) which had a Mapharsen equivalent of 2.0. This compound has therapeutic effect in yaws (12) and African trypanosomiasis (13). The other arsenical is the (*p*-carbamylphenylarsenylenedithio)diacetic acid (arsenamide), which had a Mapharsen equivalent of 0.15. This compound, also referred to as Tropical Disease Center No. 970, has therapeutic action in filariasis (13–17).

DISCUSSION

This appears to be the first record of the action against relapsing fever spirochetes *in vivo* of subtilin, methylated subtilin, bacitracin, Melarsen oxide, arsenamide and of 4,4′-bis(4,6-dihydroxy-2-*s*-triazinylamino)-2,2′-stilbenedisulfonic acid disodium salt. The activity of each of the first four agents is outstanding. Each would appear worthy of trial in human relapsing fever if activity were the only criterion to be considered. However, the decision to try any of these agents should also take into account their other pharmacologic properties. The appraisal of these properties is beyond the scope of of this study.

Although many of the compounds were inactive, these negative results should be useful to others interested in searching for new antispirochetal agents since each agent apparently was tested under conditions suitable to the detection of even slight activity. Inasmuch as the available literature indicates that most agents active against one species of spirochete also have some effect upon other species (exclusive of the leptospira), it probably is safe to presume that agents inactive in the present test would be very poor candidates for trial against other spirochetal infections.

[6] The authors are indebted to Mrs. Margaret Galbraith of these Laboratories for these antibacterial assays.

[7] Parke, Davis and Company trade mark.

SUMMARY

1. Four antibiotics and 206 synthetic compounds were compared with sodium penicillin G and Mapharsen, respectively, for their ability to suppress the spirochetemia in standardized acute infections of *Borrelia novyi* in mice.

2. Subtilin, methylated subtilin, and bacitracin were highly effective. Streptomycin was slightly active. Penicillin G and bacitracin acted synergistically.

3. Two of the active synthetic compounds were arsenicals. These were Melarsen oxide, which was highly effective, and arsenamide, which was slightly active. Only 2 of the nonmetallic synthetic compounds had discernible effect. These were germanin and 4,4′-bis(4,6-dihydroxy-2-*s*-triazinylamino)-2,2′-stilbenedisulfonic acid disodium salt, but neither was highly active.

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Cardiac Activity of Unsaturated Lactones as Related to Their Theoretical Peroxide Formation*,†

By DUANE G. WENZEL‡ and MORENO L. KEPLINGER

A study has been made of a group of gamma lactones and peroxides on the frog heart. The hypothesis presented to explain the experimental results states that the cardiac activity of the lactones, as tested, is based upon peroxide formation from a free radical which in turn necessitates a resonating structure. Results are given indicating that the cardioactive product may be a hydroperoxide rather than the carbon to carbon form.

THE POSITIVE inotropic action exerted by the cardiac glycosides has been shown to be dependent upon the presence of an unsaturated lactone moiety (1–5). This observation initiated several attempts to evaluate the cardiac activity of simple lactones. Utilizing the frog lymph sac toxicity method, Chen, *et al.* (6), could not find any digitalis-like action in four lactones tested. Later, however, this same group was able to cause systolic standstill using the intact frog heart (7). It should be noted that these lactones have no digitalis-like action on the mammalian heart (7, 8). The α,β-angelica lactone actually produces "cardiac depression" of the cat heart and eliminates irregularities induced by digitalis or barium (9).

Krayer and co-workers obtained positive results through the use of the isolated frog heart made hypodynamic by a low calcium perfusion fluid (10, 11). They observed that these lactones were considerably more potent when the perfusion mixture was saturated with oxygen, while activity disappeared with the substitution of nitrogen. It was also noted that the lactones which were not oxidized (dimethylascorbic acid and dehydroascorbic acid) likewise did not possess cardiac acitivity. Furthermore, the presence of hydrogen peroxide was demonstrated in solutions of ascorbic acid eliciting a positive inotropic response. On the basis of this evidence and the fact that certain peroxides *per se* have been shown to cause a systolic action on the frog heart (12), Krayer, *et al.*, postulated that the formation of peroxides is responsible for the cardiac action of the lactones (11).

By use of a peroxide indicator, Mendez (13) demonstrated the presence of peroxides in perfusion solutions of α,β- and β,γ-angelica lactones. The β,γ-form, which produces a greater cardiac activity also has a higher peroxide content. He initially attributed the formation of peroxides to the presence of metallic impurities, as the peroxide effect was blocked by sulfhydryl compounds. In a later work (14) he observed a systolic action of sulfhydryl reagents indicating that the peroxides may produce their positive inotropic effect by an oxidative blocking of sulfhydryl enzymes.

Giarman utilized both the cardiotoxic and the cardiotonic methods for testing a group of gamma and delta lactones and established that γ-lactones with δ-2 unsaturation or with δ-1 unsaturation accompanied by a system of conjugated double bonds were the most effective (15, 16). Saturation of the lactones abolished cardiac activity and alkyl substitution was observed to reduce it.

Polarographic measurements indicate β,γ-angelica lactone to have an oxygen uptake several times that of the α,β-isomer (17), a difference previously observed by Mendez (13) with peroxide indicators and upon the frog heart. In the polarographic determinations it was noted that the lactone took up no oxygen in an acidic solution. This confirms the work of Clark (18) who found that the cardiac drugs are inactive if the perfusion solution has a pH of less than 6.4. Other polarographic studies have demonstrated that isomeric lactones such as α,β- and β,γ-angelica lactones form the same peroxide which appears to be a hydroperoxide (19).

The purpose of this investigation was to determine if some unsaturated lactones do not have a digitalis like action on the frog heart and if their activity may be predicted on the basis of potential peroxide formation. It was also decided to explore the type of peroxide producing this effect by testing both hydro- and carbon to carbon peroxides.

EXPERIMENTAL

Materials.—Tertiary-butyl hydrogen peroxide and tertiary-butyl perbenzoate are products of the Lucidol Division, Novadel-Agene Corporation, Buffalo, N. Y. The 2,2-bis-butane peroxide and ditertiary butyl peroxide are produced by the Shell

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Development Corporation, Emeryville, Calif. Ouabain U. S. P. was obtained from S. B. Penick and Company.

The lactones used in this study were prepared by Dr. Calvin A. Vanderwerf and his associates of the Department of Chemistry, University of Kansas.

General Methods.—The experiments were carried out in the summer of 1952 on large frogs of the species *Rana pipiens*. The frogs were prepared by pithing and exposing the heart. A modified Greene cannula with an upright overflow arm of 6 cm. and an internal diameter of 3.5 mm. was tied into the vena cava for an inflow. The cannula was made with the tip turned upward so as not to slip out of the vena cava when the heart was pulled up for recording. A simple ninety-degree cannula with the upright outflow arm of the same dimensions was inserted in the left aortic branch. The right branch was ligated. This procedure maintained a constant hydrostatic pressure on the heart. The inflow cannula was connected by polyethylene tubing to dropping bottles of the type used for the clinical administration of fluids. The stoppers were from Venopak kits which allowed the fluid to flow dropwise when controlled by a pinchclamp. The rate of flow was maintained at 2–2.5 cc. per minute. A spray of the Ringer's solution used for the perfusion was directed on the heart at all times from a curved fine-tipped tube attached to a dropping funnel of the fluid.

The normal perfusion fluid was Ringer's solution as used by Krayer (10) and contained NaCl, 0.65%; KCl, 0.014%; $CaCl_2$, 0.011%; $NaHCO_3$, 0.02%. The concentration of all salts was the same in the low-calcium solution except $CaCl_2$ which was decreased to one-half the normal concentration or 0.0055%.

One per cent stock solutions were prepared of the lactones and peroxides in 95% ethanol. The concentration of ethanol in the test solutions was never greater than 1%. In control experiments this concentration produced no demonstrable change in the action of the drugs.

The *p*H of the perfusion solutions of the drugs varied between 7.5 and 7.8. Contractions of the heart were recorded by means of a fine pinhook inserted into the tip of the ventricle. This was connected by a light thread to a gravity recording lever.

PROCEDURE

Cardiotoxic.—The technique employed was similar to that used by Giarman (15). Four frogs were used for each drug tested. Normal Ringer's solution was perfused through the heart until a constant rate and amplitude were obtained. At this point a 1:10,000 solution of the drug in normal Ringer's solution was substituted. The heart rate was recorded during the period of uniform beating and again toward the end of the experiment. The time from the administration of the drug to cardiac standstill and the type of standstill were obtained.

Cardiotonic.—The method used was that described by Giarman (16). The heart was perfused with normal Ringer's solution until the rate and amplitude became constant. Low-calcium Ringer's solution was substituted for the normal solution and perfusion continued until a hydrodynamic state ensued. The normal solution was again perfused to determine if the heart could be restored to the original amplitude. The low-calcium medium was again perfused until hypodynamia occurred and finally the low-calcium solution containing the drug was given.

Various concentrations of the drug were tested in an attempt to find the liminal positive inotropic concentration. This has been defined by Giarman (16) as the smallest concentration of the drug which will restore the ventricular amplitude of three out of four hypodynamic hearts to 75% or more of their normal amplitude within twenty minutes. A comparison of the compounds studied was obtained by establishing a potency ratio between the liminal positive inotropic millimolar concentrations of ouabain and that of the test drugs.

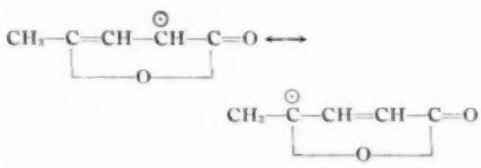
RESULTS

The cardiotoxic and cardiotonic effects of the lactones and peroxides tested are shown in Table I and Figures 1–6.

Giarman (15) emphasized the greater activity of lactones with δ-2 unsaturation over those having the double bond in the 1 position, and essentially concluded that while δ-2 unsaturation was most effective in producing a digitalis-like cardiac toxicity, unsaturation in the 1 position produced an active compound if it was incorporated in a conjugated system of double bonds. While this observation is factually correct, it does not account for the basic mechanism which causes the difference in activities of isomeric lactones such as α,β- and β,γ-angelica lactones. Further it does not explain why certain of the lactones induce systolic standstill and restore the amplitude of the hypodynamic heart while other similar compounds stop the heart in diastole and have no restorative value.

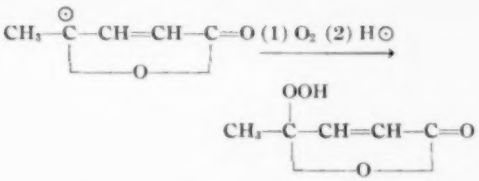
The following hypothesis,[1] based upon the experimental evidence and the studies of Dr. Calvin A. Vanderwerf, apparently explains the mechanism by which the lactones induce their cardiac activity.

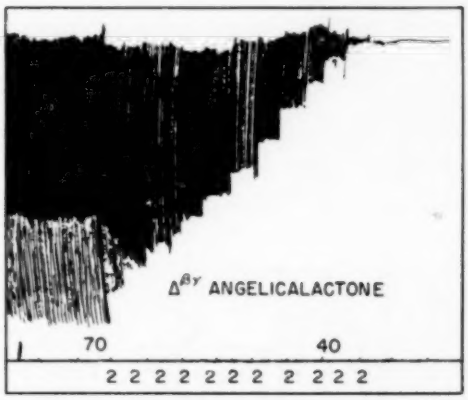
In the case of α,β- and β,γ-angelica lactones, the difference in their activities (Table I; Figs. 1 and 2) and a similar difference in the rate of oxygen uptake, as demonstrated polarographically (17) can be explained by the hypothesis that peroxide formation proceeds via a free radical. This radical is formed by abstraction of a hydrogen atom from a molecule of the original lactone and must be somewhat stabilized through resonance. The contributing resonance structures for the free radical formed from both α,β- and β,γ-angelica lactones, for example are postulated as follows:

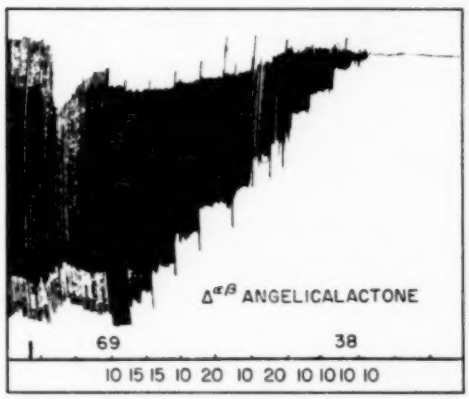


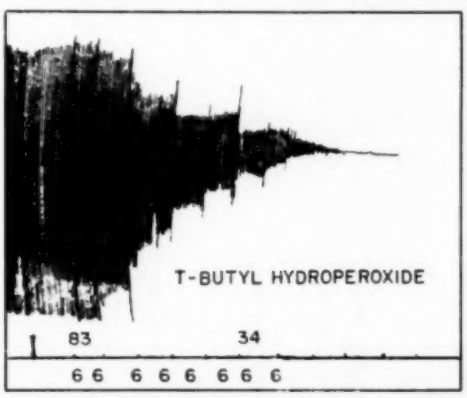
The peroxide is then pictured as being formed by addition of O_2 to the radical followed by addition of a hydrogen atom.

[1] This hypothesis will be further elaborated in a publication by Kubitz, *et al.*









Figs. 1, 2, and 3.—Cardiotoxic action. Drug replacement at sign. Time is recorded at ten-second intervals. Figures above the line indicate heart rate per minute. Those below are periods of drum arrest.

It is significant that in all cases where such resonance can occur the hypothesis predicts that the same intermediate radical, and therefore the same peroxide, will be formed from any isomeric pair of α,β- and β,γ-unsaturated lactones. Polarographic data have shown this to be the case (17). The rate of formation of the radical and therefore the ultimate rate of formation of the hydroperoxide, if the first step is rate-controlling, may vary considerably for any pair of isomers, being faster for that lactone of the pair which is thermodynamically less stable. In the case of α,β- and β,γ-angelica lactones the least stable compound is the β,γ-isomer which therefore forms the peroxide more rapidly and induces a more rapid cardiac stoppage (Table I; Figs. 1 and 2).

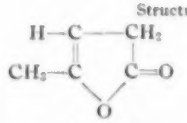
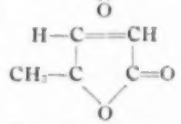
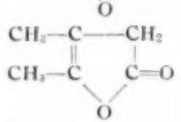
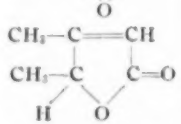
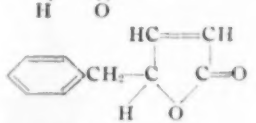
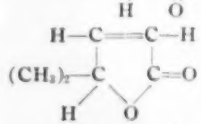
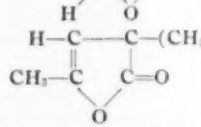
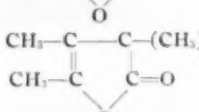
While the position of the double bond may account for the difference in activity of an isomeric pair, it does not account for the fact that certain of the lactones tested, as indicated in Table I, do not induce a digitalis-like systolic stoppage but cause what may be regarded as a strictly toxic phenomenon of diastolic stoppage. These same systolically inactive compounds were also ineffective in restoring the amplitude of the hypodynamic heart. It has also been demonstrated polarographically (19), using the same biological fluid as in this experiment, that they do not take up oxygen. From the previous hypothesis, any structural feature which renders resonance in the radical impossible or extensively inhibits it will prevent peroxide formation. Thus di-substitution on either the α- or γ-carbon, such as in γ-methyl-$\Delta^{\alpha,\beta}$-angelica lactone, α,α-dimethyl-$\Delta^{\beta,\gamma}$-angelica lactone and α,α,β-trimethyl-$\Delta^{\beta,\gamma}$-angelica lactone which would preclude the possibility of the shift in the double bond necessitated by resonance, effectively prevents peroxide formation. Experimentally, the compounds mentioned which possess these structural features did not produce systolic stoppage nor increase the amplitude of contraction of the hypodynamic heart (Table I).

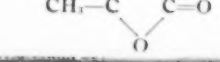
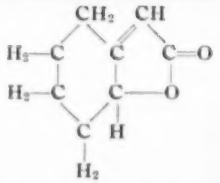
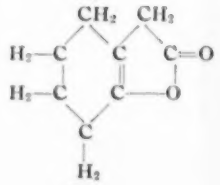
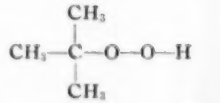
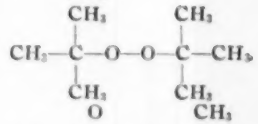
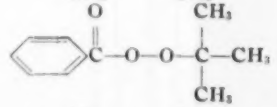
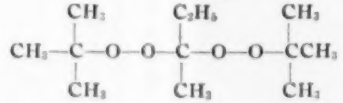
The same explanation may also account for the failure of the γ-lactone of 2-hydroxy cyclohexylidene acetic acid and the γ-lactone of 2-hydroxy-1-cyclohexen-1-yl acetic acid to show typical activity as indicated in Table I. Here, because of the fused ring system, resonance may be extensively inhibited by virtue of the fact that the coplanarity required in a conjugative-type resonance system cannot be achieved, due to an excessive strain which would exist if all atoms of the two rings were to be forced into the same plane.

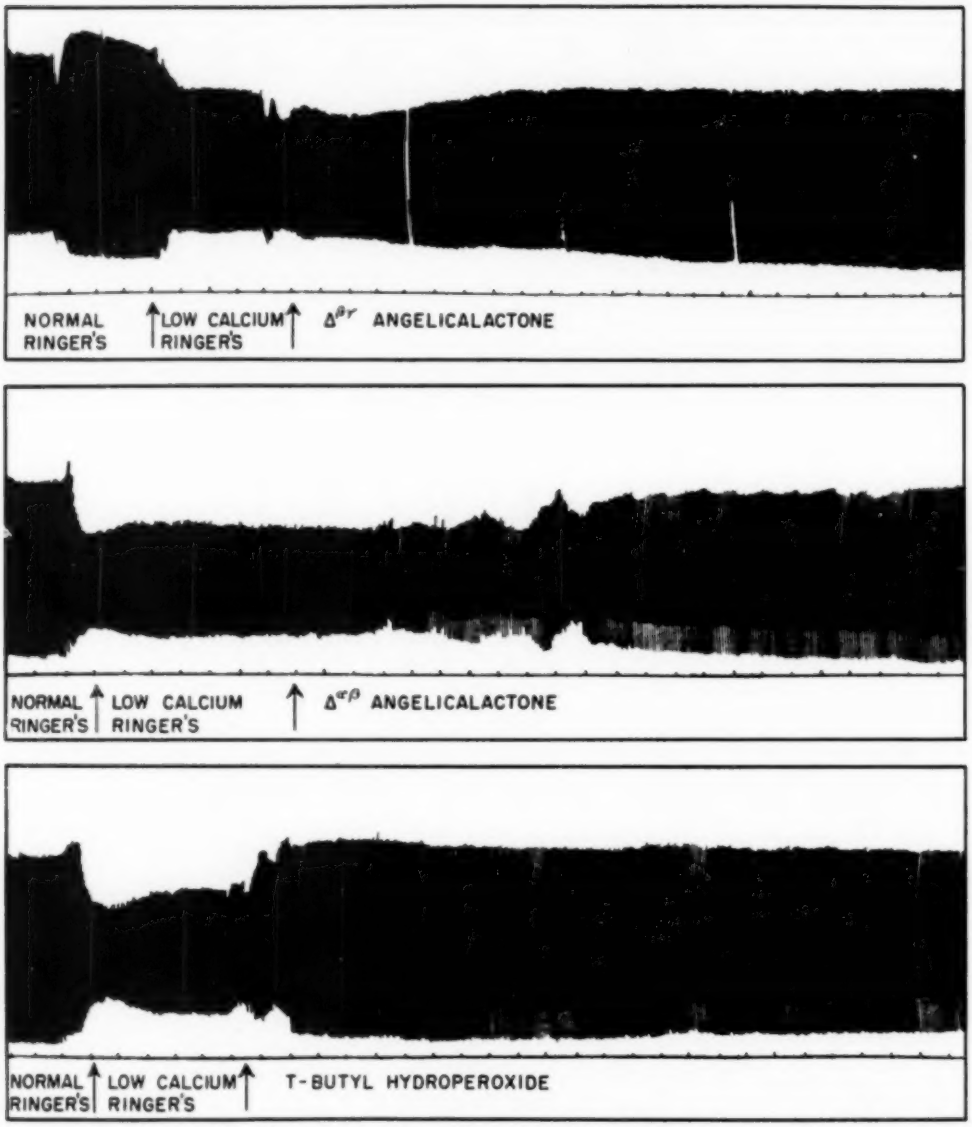
(I)

The apparently anomalous situation observed by Giarman (15) in the marked cardiac activity of the dilactone of pulvinic acid may also be explained on the basis of this theory. This compound has a highly conjugated system with δ-1 unsaturation of the γ-lactone rings and would thus appear to be low in predictable peroxide-forming activity. Its effectiveness may be attributed, however, to the resonance stabilization of the radical (I) formed by removal of a hydrogen atom from the *ortho*- or *para*-carbon of

TABLE I.—TOXICITY AND CARDIOTONIC ACTIVITY

Compound	Structure	Mean Time to Cardiac Arrest, Minutes	Remarks	Liminal Positive Inotropic Concentration		Potency Ratio Ouabain = 1
				Mg./%	mM.	
$\Delta^{\beta,\gamma}$-Angelica lactone		29	Systolic standstill; amplitude increased; rate slowed	1.25	0.128	600
$\Delta^{\alpha,\beta}$-Angelica lactone		142	Systolic standstill; amplitude increased; rate slowed	10.00	1.020	4,770
β-methyl $\Delta^{\beta,\gamma}$-Angelica lactone		158	Systolic standstill; no change in amplitude; rate slowed	10.00	0.877	4,098
β-methyl $\Delta^{\alpha,\beta}$-Angelica lactone		102	Systolic standstill; amplitude increased; rate slowed	10.00	0.877	4,098
δ-phenyl $\Delta^{\alpha,\beta}$-Angelica lactone		17	Systolic standstill; amplitude decreased; rate slowed	5.00	0.287	1,341
γ-methyl $\Delta^{\alpha,\beta}$-Angelica lactone		404	Stopped in diastole; amplitude decreased; no change in rate	>100	...	...
α,α-dimethyl $\Delta^{\beta,\gamma}$-Angelica lactone		201	Stopped in diastole; amplitude decreased; no change in rate	>100	...	...
α,α,β-trimethyl $\Delta^{\beta,\gamma}$-Angelica lactone		70	Stopped in diastole; amplitude decreased; rate slowed	>100	...	...

$\Delta^{\alpha,\gamma}$-Angelica lactone			rate slowed		
γ-lactone of 2-hydroxy cyclohexylideneacetic acid		291	Stopped in diastole; amplitude decreased; rate slowed	>100	...	...
γ-lactone of 2-hydroxy-1-cyclohexen-1-yl acetic acid		214	Ventricle size somewhat reduced; amplitude increased; rate slowed	>100	...	...
Tertiary-butyl hydroperoxide		48	Systolic standstill; no change in amplitude; rate slowed	2.00	0.222	1,037
Di-tertiary-butyl peroxide		63	Stopped in diastole; amplitude decreased; no change in rate	...	...	...
Tertiary-butyl perbenzoate		24	Stopped in diastole; amplitude decreased; no change in rate	...	...	...
2,2-bis (tertiary-butylperoxy) butane		21	Stopped in diastole; amplitude decreased; no change in rate	...	...	...
Ouabain	.....	20	Systolic standstill; amplitude increased; rate slowed	0.0125	0.000214	1



Figs. 4, 5, and 6.—Cardiotonic action. Time is recorded at forty-five-second intervals.

either benzene ring. Many resonance structures may be written for the radical, and although no one of them would account for the activity, the total contribution of all would be expected to induce stability.

From chemical analysis (19) it appears that the type of peroxide formed by lactones is a hydroperoxide. Confirmatory evidence is offered in the activity of tertiary-butyl hydroperoxide which produces systolic arrest and an increased amplitude of the hypodynamic heart (Figs. 3 and 6) while three carbon to carbon peroxides tested were inactive cardiotonically.

SUMMARY

1. Ten gamma lactones and four peroxides were tested on the frog heart *in situ*.

2. An hypothesis was advanced to explain the pharmacological results and the polarographic studies of others. This postulate states that the activity of the lactones on the frog heart depends upon their ability to form a free radical through resonance and thence a cardioactive peroxide.

3. Evidence is presented indicating the type of peroxide formed to be a hydroperoxide.

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The Preservation of Parenteral Vegetable Oils by Chemical Agents*

By PHILIP C. EISMAN, D. JACONIA, and R. L. MAYER

Solutions in sesame oil of a number of commonly used preservatives, as well as several others known to have bactericidal properties, were ineffective in destroying *B. subtilis* spores under anhydrous conditions. Similarly, seven commercial samples of "preserved" parenteral vegetable oils were found to be without sporicidal activity.

ACCORDING TO U. S. P. XIV, all injectable oils which are marketed in multiple-dose vials are to be rendered bacteriostatic by the addition of suitable chemical preservatives (1). Since there is very little practical information in the literature dealing with chemical disinfectants for such products, a study was initiated for the purpose of selecting an effective oil-soluble preservative.

It is a generally accepted fact that vegetable oils, in the absence of water, do not permit the growth of microorganisms and it therefore may be considered that such anhydrous oils possess intrinsic bacteriostatic properties.

Bullock and Keepe (2) have shown that in the dehydrated state *Strep. fecalis* and *B. subtilis* spores remain viable in peanut oil for more than six months. Although other reports (3, 4) point to the relatively rapid destruction of bacteria suspended in oils commonly used as vehicles for injections, the possibility of contamination of parenteral oils in multiple-dose containers with pathogenic organisms, particularly of the spore-forming type, and the subsequent injection of such material into patients deserves serious consideration.

Since the rate of autodisinfection is variable and unpredictable, depending upon a number of factors, the addition of a preservative to the finished product, as now required according to the U. S. P., should theoretically bring about a complete and rapid destruction of all contaminating organisms. It is not sufficient that the incorporation into the oil of a chemical agent should merely *prevent* the growth of all microorganisms without regard to their actual destruction.

In view of these considerations, a variety of substances were examined for their effectiveness in sterilizing experimentally contaminated sesame oil, particularly under anhydrous conditions. Sesame oil was selected as the test oil since it is widely used and has been shown to be the most suitable from a pharmacologic standpoint, of the more commonly employed parenteral oils (5).

EXPERIMENTAL

Survival of Organisms in Sesame Oil in Presence of Small Amounts of Water

Contamination of the contents of multiple-dose vials may at times occur as a result of the introduction of droplets of nonsterile water through the syringe needle. Preliminary experiments however, to contaminate samples of oil with droplets of distilled water containing the test organisms (*Pseudo. aeruginosa* and *Staph. aureus*), proved unsuccessful

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because these organisms succumbed within twenty-four hours. When nutrient broth was substituted for the distilled water, considerable growth was obtained as is clearly indicated by the results of a typical experiment shown in Table I. A sample of the same oil containing a merthiolate concentration of 1:50,000 was tested for bactericidal activity. Bottles containing 2.0-ml. amounts of sterile sesame oil were each seeded with 0.5 ml. of a 1:100 dilution of an overnight culture of *Ps. aeruginosa* or *Staph. aureus*. In the *B. subtilis* tests, 0.05 ml. of a pasteurized suspension containing approximately 50,000 viable spores was added to each sample of oil. After the introduction of the test organisms, the bottles were shaken vigorously by hand and then stored in the dark at room temperature. At intervals within a ten-day period, 98 ml. of sterile 0.1% aqueous solution of Tween 80 were added to each bottle of oil, the contents shaken thoroughly, and plated for bacterial count.

Preparation of Spore-Coated Threads.—Sterile, white surgical silk thread (No. 2 size) of 0.5-inch lengths were immersed in a pasteurized *B. subtilis* spore suspension (200,000,000 per ml.) for one hour. The spore-coated threads were then dried overnight in a desiccator over $CaCl_2$ under normal atmospheric pressure.

Preservatives Tested.—The substances tested in this study and listed below are soluble in sesame oil. They consisted of the following preservatives commonly used in the pharmaceutical industry for aqueous preparations, as well as several chemical agents of academic interest: phenol, catechol, resorcinol, hexylresorcinol, *m*-cresol, *p*-benzyl phenol, thymol, carvacrol, *o*-chlorophenol, 2-bromo-4-phenyl phenol, salicylic acid, propyl-*p*-hydroxybenzoate, benzyl alcohol, chlorobutanol (chloretone), epichlorhydrin (chloropropylene oxide), epibromhydrin, phenyl mercuric borate, and ethylene oxide.

TABLE I.—VIABILITY OF TEST ORGANISMS IN PRESERVED AND UNPRESERVED SESAME OIL—BROTH INOCULUM

Preservative	Test Organism	Numbers of Surviving Organisms (Per Ml. of Oil) After Contact for Specified Time: At Start	1 Day	4 Days	6 Days	8 Days
None (unpreserved)	*Ps. aeruginosa*	76,000	...	527,000,000	482,000,000	476,000,000
	Staph. aureus	46,600	25,000	24,000,000	68,000,000	25,000,000
	B. subtilis spores	46,500	25,000	10,000	8,000	2,300
Merthiolate 1:50,000	*Ps. aeruginosa*	76,000	<100	<100	<100	...
	Staph. aureus	46,600	<100	<100	<100	...
	B. subtilis spores	46,500	68,000	29,500	11,000	10,600

Results.—Considerable growth of *Ps. aeruginosa* and *Staph. aureus* had taken place in the unpreserved sesame oil seeded with a very small volume of diluted broth culture, but these organisms were rapidly destroyed in sesame oil containing 1:50,000 merthiolate. Viable *B. subtilis* spores, on the other hand, slowly diminished in numbers when suspended in unpreserved oil and were not completely destroyed by the eighth day in the presence of merthiolate. Using the same test procedure, 1.0 and 2.0% ethyl alcohol, as well as 2.0% benzyl alcohol, were also capable of rapidly destroying the two nonsporulating test organisms but were ineffective against the spores.

Survival of B. subtilis Spores in Preserved and Unpreserved Sesame Oil under Anhydrous Conditions

Since it is also possible that contamination of vials may occur from the use of a nonsterile, dry syringe needle, it was felt desirable to find a chemical agent capable of sterilizing sesame or other vegetable oils in the relatively complete absence of water.

Initial experiments to evenly disperse dried, nonsporulating test organisms such as *E. coli*, *Ps. aeruginosa*, and *Staph. aureus* throughout the oil in the total absence of water were unsuccessful; moreover, these organisms very rapidly succumbed under these conditions. We therefore resorted to a modification of the old but still useful "silk thread spore test" which essentially involves the placing of a number of standard lengths of silk thread, coated with dried, viable *B. subtilis* spores, into portions of preserved sesame oil. At intervals throughout a thirteen-day period, threads were removed and the numbers of surviving spores determined by plating.

Measurement of Sporicidal Action.—Fifteen spore-coated threads were placed in 10-ml. amounts of the oil containing varying concentrations of the test substances. The flasks were kept at room temperature in the dark for thirteen days. At varying intervals of time, two threads were removed and one was placed in a bottle containing 100 ml. of sterile aqueous 0.1% Tween 80 solution and thoroughly shaken. The spore count of 1.0- and 0.1-ml. amounts of this solution was then determined by agar plating. Initial tests had shown that the Tween 80 solution *per se* had no sporicidal activity. The second thread was placed into 15 ml. of thioglycollate medium. The agar plates and tubes of broth were incubated at 37°.

Table II records the sporicidal effectiveness of the test substances as indicated by the survivor counts at varying intervals of time.

According to the data presented in Table II, ethylene oxide, at the concentration tested (1.0%), was the only substance capable of completely sterilizing silk threads within a twenty-four-hour period. None of the other chemical agents investigated exerted a rapid, sterilizing effect. Selecting the third day of exposure as an arbitrary basis for evaluating sporicidal effectiveness, it is evident that among these substances, known to be active in an aqueous environment, only four were significantly bactericidal in moisture free sesame oil: catechol, 1.0%; hexylresorcinol, 0.5%; carvacrol, 1.0%, and epichlorhydrin, 1.0%. The least active compounds were propyl-*p*-hydroxybenzoate (1.0%), chlorobutanol (5.0%), and benzyl alcohol at concentrations below 2.0%.

In view of the relatively poor sporicidal activity in sesame oil of a number of accepted and well-known

TABLE II.—SPORICIDAL ACTIVITIES IN SESAME OIL OF A NUMBER OF SUBSTANCES INCLUDING COMMONLY USED PRESERVATIVES—ANHYDROUS CONDITIONS

Preservative	Concentration, %	Numbers of Surviving Spores After Contact for Specified Time in Sesame Oil Solutions of Commonly Used Preservatives				
		24 Hr.	3 Days	7 Days	10 Days	13 Days
None (controls)	1st Test	28,000	33,000	...	33,000	...
	2nd Test	10,900	32,000	3,800	...	4,900
	3rd Test	60,000	41,000	37,800	12,500	3,000
	4th Test	30,800	19,000	19,400	24,000	4,400
Phenol	2.0	30,700	3,150	0	(0)[a]	(0)
	2.0	12,400	2,800	100	–	(0)
	1.0	22,500	4,900	0	(0)	(0)
	1.0	28,300	10,900	4,000	–	100
	0.5	40,000	39,950	0	(0)	(0)
	0.5	30,000	20,500	4,500	800	200
	0.5	14,800	8,900	600	100	(0)
	0.25	30,700	8,800	0	(0)	(0)
	0.25	25,900	15,000	2,450	–	0
	0.125	27,100	20,400	0	0	–
	0.125	14,900	13,000	7,500	–	300
Catechol	1.0	36,800	900	0	0	0
	0.5	15,600	2,500	100	0	0
Resorcinol	1.0	4,700	3,500	2,050	200	100
	0.5	6,500	4,500	2,000	4,000	200
Hexylresorcinol	0.5	4,600	800	100	0	0
	0.25	6,300	1,400	150	0	0
m-Cresol	1.0	17,300	4,900	4,500	–	0
	0.5	30,400	14,200	6,450	–	0
	0.1	26,700	4,900	2,750	–	400
p-Benzyl phenol	1.0	16,100	15,600	1,300	–	0
	0.5	13,900	16,500	0	–	0
	0.1	25,700	19,200	3,300	–	5,200
Thymol	1.0	8,100	2,700	100	0	0
	0.5	13,000	13,300	400	0	0
Carvacrol	1.0	1,600	300	0	0	0
	0.5	4,600	5,700	400	0	0
o-Chlorophenol	1.0	8,000	3,600	400	–	0
	0.5	21,300	9,300	3,000	–	0
	0.1	32,800	20,900	10,500	–	1,600
2-Bromo-4-phenyl-phenol	1.0	31,600	17,300	9,100	1,100	200
	0.5	37,000	8,800	18,000	4,800	4,800
Salicylic acid	1.0	5,500	19,200	1,500	100	100
	0.5	7,000	14,400	3,400	300	800
Propyl-*para*-hydroxybenzoate	1.0	21,400	27,300	32,200	42,000	4,200
	0.5	14,900	33,000	97,000	58,000	24,000
	0.5	43,200	57,000	10,700	–	6,000
	0.1	75,000	40,000	29,700	–	4,000
Benzyl alcohol	5.0	19,700	5,100	(0)	(0)	(0)
	3.0	23,800	16,200	100	(0)	(0)
	2.0	24,800	9,450	800	(0)	(0)
	1.0	27,800	23,500	6,900	26,900	2,200
	0.5	10,600	4,600	15,200	16,500	4,000
	0.25	24,800	15,600	12,750	12,100	2,000
	0.125	26,500	11,750	11,650	2,400	(0)
Chlorobutanol (chloretone)	5.0	18,400	30,700	27,200	–	19,900
	3.0	33,200	23,000	14,400	–	22,900
	2.0	30,500	24,000	28,000	–	36,000
	1.0	26,700	10,800	21,500	–	10,800
	1.0	42,200	19,400	43,000	30,000	8,400
Epichlorhydrin (chloropropylene oxide)	1.0	20,100	100	0	0	0
	0.5	9,000	2,400	100	0	0
	0.1	18,900	6,500	3,200	300	100
Epibromhydrin	1.0	38,000 (1 Hr.)	1,000	0	100	0
	0.5	8,200 (1 Hr)	2,700	600	0	0
	0.1	11,300 (1 Hr)	3,700	800	3,500	800
Phenyl mercuric borate	0.01	15,700	5,800	12,300	5,700	4,600
	0.002	23,500	13,200	17,400	8,900	1,900
		1 hr.	5 hr.	24 hr.	4 days	
Ethylene oxide	1.0	49,000	200	(0)	(0)	...

[a] (0) indicates that a duplicate thread placed in thioglycollate broth proved to be sterile.

TABLE III.—SPORICIDAL ACTIVITIES OF SEVEN COMMERCIAL SOLUTIONS OF HORMONES IN PRESERVED VEGETABLE OILS

Commercial Product	Oil Used as Vehicle	Preservatives	Number of Surviving Spores After Contact for Specified Time			
			24 Hr.	4 Days	9 Days	14 Days
A	Sesame	3.0% chlorobutanol	10,000	112,000	75,000	84,000
B	Sesame	0.5% chlorobutanol	39,000	75,000	50,000	44,000
C	Sesame	0.5% chlorobutanol and 2.0% benzyl alcohol	55,000	70,000	33,000	22,500
D	Persic	3.0% benzyl alcohol	60,000	45,000	4,000	0
E	Sesame	0.1% propyl *p*-hydroxybenzoate and 3.0% benzyl alcohol	140,000	115,000	15,000	7,500
F	Sesame	1:50,000 phenyl mercuric borate	30,000	50,000	18,000	22,500
G	Vegetable	0.5% cresol	181,000	132,000	1,000	0
G	Vegetable	0.5% cresol	80,000	99,000	30,000	22,300

preservatives, as shown in Table III, it became of interest to examine commercially available samples of therapeutic agents in "preserved" parenteral oils.

The results obtained with seven products tested by the silk thread method are shown in Table III.

Results.—It is apparent from Table III that none of the seven commercial samples tested was capable of appreciably destroying *B. subtilis* spores. A limited degree of sporicidal activity was noted with samples *D*, containing 3.0% benzyl alcohol and *G*, preserved with 0.5% cresol. Upon retesting sample *G* however, this slight activity was not substantiated.

DISCUSSION

A substance, to be considered suitable as a preservative for injectable oils, should be bactericidal in the absence of water since it is possible that contamination may result from the use of dry but unsterile syringe needles. For this reason, one of the methods selected for evaluating the efficacy of preservatives involved a nonaqueous system. Since microorganisms are unable to grow in anhydrous vegetable oils, the addition of a bacteriostatic agent is meaningless unless it is capable, at the concentration used, of exerting a bactericidal action. According to the results obtained by the silk thread spore method, none of the compounds generally used as preservatives in the pharmaceutical industry was effective in bringing about a reasonably rapid destruction of *B. subtilis* spores. Of the other chemical substances evaluated, ethylene oxide exerted a very rapid and complete destruction of spores within twenty-four hours, regardless of other properties governing its use as a parenteral oil preservative.

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The Composition of Honey Locust Gum*

By ERNEST ANDERSON† and BEVAN B. BLAKE‡

THE HONEY LOCUST TREE, *Gleditschia triacanthos*(L) is a native of the Central United States. It is a leguminous tree, 75 to 140 feet in height, and 2 to 3 feet in diameter. The seeds have an endosperm mucilage which is a Mannogalactan (1) and a normal product of the growth of the tree. The gum, on the other hand, is a pathological product and is found only on injured or diseased trees. There is no relation in composition or structure between the two products.

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PROCEDURE

Source.—The gum used was collected from trees growing near Tucson. It consists of lumps or crusts varying from one-fourth inch to one inch in thickness and covering a square inch or more of surface. It was exuded in a soft condition and hardened afterwards. It varies from a light honey color to a dark color.

Purification.—In purifying the gum hot water should not be used. When the powdered gum is allowed to stand with 20 times its weight of water at ordinary temperature and is then stirred it soon dissolves. This solution can be filtered, at the pump, through filter paper. However, if the solution is heated to boiling it filters only with great

difficulty. When the clear filtrate is added slowly, with stirring, to 10 times its volume of 90% ethanol, it forms a colloidal solution which is readily flocculated by addition of a few cc. of a saturated sodium chloride solution. After settling, the supernatant liquid is siphoned off and the precipitated gum is centrifuged and filtered off and washed with ethanol and ether and dried *in vacuo*. The recovery is 85% of a light colored product.

Preparation of the Gum Acid.—Pure gum weighing 11.3 Gm. was dissolved in 50 cc. of water at room temperature. To this solution was added a cold solution of 14 cc. of concentrated hydrochloric acid which had been diluted with an equal volume of water. The resulting solution was poured slowly, with stirring, into 95% ethanol. The gum acid separated as a flocculent precipitate which collected together and was removed as a lump on a stirring rod and placed in 95% ethanol. After it had been rubbed to a powder, it was filtered, at the pump, and washed with ethanol and ether and dried *in vacuo*. The yield of pure gum acid was 85% of the gum used.

Analysis of the Gum.—The presence of a uronic acid was established by the naphtho-resorcinol test (2, 3) and by the evolution of carbon dioxide when the gum was heated in a 12% solution of hydrochloric acid (4). Tests for methoxyl groups by the methods of Denigès (5), and von Fellenberg (6), were negative.

The results of the analyses of the crude gum, the purified gum, and the gum acid are given in Table I. The methods used are standard and are fully described in the literature (2, 7, 8). The results are corrected for moisture and ash. Since it is shown below that L-arabinose and D-galactose are the only sugars present, the percentages of pentosan and hexosan are reported in Table I as L-araban and D-galactan.

TABLE I.—ANALYSIS OF UNPURIFIED AND PURIFIED HONEY LOCUST GUM[a] AND GUM ACID

	Crude	Pure	Acid
Moisture, %	9.02	6.10	3.35
Ash, %	3.64	4.28	0.41
Carbon dioxide, %	4.99	4.83	5.07
Uronic acid, %	21.89	21.19	22.24
L-Araban, %	46.61	45.57	43.90
D-Galactan, %	31.50	33.23	33.86
Equivalent weight	882.00	911.00	868.00
Araban units per uronic acid	3.12	3.15	2.89
Galactan units per uronic acid	1.72	1.87	1.82
$[\alpha]_D^{25}$°	−11.42°	−10.60°	−10.99°

[a] The results have been corrected for moisture and ash. The equivalent weights are calculated from the per cent uronic acid. The per cent galactan was calculated as the difference between 100 and the sum of the percentages of uronic acid and araban.

The analyses show that in crude and purified samples of the gum there is an average of slightly more than three araban units to each uronic acid while in the free gum acid there are slightly less than three araban units to each uronic acid. This indicates that during the preparation of the free acid a slight amount of L-arabinose was split off. This is not surprising since the arabinose was found to be easily liberated in warm acid solution. The analyses also show that the number of D-galactan units in the crude and purified gum and the free gum acid were on the average slightly less than two for each uronic acid group.

Hydrolyses of the Gum.—Several lots of the gum and its salts were hydrolyzed at different times under varying conditions. In all of these, weighed amounts of the material were mixed with known volumes of water and weight of sulfuric acid and the mixture was heated for a known length of time and temperature. The hot solutions were then neutralized by addition of barium carbonate, decolorized with activated carbon, filtered at the pump, and the filtrates were concentrated *in vacuo* to syrups. The barium salts were precipitated by addition of the syrups to ethanol. They were further purified by redissolving them in water and again adding the solutions to ethanol. The alcohol solutions of the sugars, free of salts, were concentrated *in vacuo* to syrups. In this way the sugars were separated from the salts which are described below.

Barium Salt A.—This salt was prepared by heating 25 Gm. of pure gum for one hour at 65° in a 2% solution of sulfuric acid. The results were 3.5 Gm. of gum sugar, which was largely L-arabinose with traces of D-galactose, and 18 Gm. of Salt *A*. The analyses of Salt *A* (Table II) shows that under these mild conditions some of both the sugars were liberated.

Barium Salt B.—This salt was prepared by heating 30 Gm. of pure gum for three hours at 80° in 4% solution of sulfuric acid. The results were 13 Gm. of gum sugar and 12 Gm. of Salt *B*. The gum sugar was largely L-arabinose with considerable D-galactose. The analysis of Salt *B* (Table II) shows that under these more rigorous conditions nearly all of the L-arabinose is liberated but one molecule of D-galactose remains with the uronic acid.

Barium Salt C.—This salt was prepared by heating 16 Gm. of Salt *B* for two hours in 100° in a 4% solution of sulfuric acid. The results were 2.5 Gm. of crystalline D-galactose and 10 Gm. of Salt *C*. The analysis of Salt *C* shows that within the limits of experimental error, all the L-arabinose has been removed and that Salt *C* is the barium salt of an aldobionic acid composed of the uronic acid combined with D-galactose.

Barium Salt D.—Aldobionic acids composed of a uronic acid and an aldose sugar, which are obtained during the hydrolysis of plant gums and mucilages have one aldehyde group free and the other combined. Hypoiodite oxidation of the reducing group of the aldobionic acid has shown that the bound aldehyde is always that of the uronic acid (8 *p* 341) (9). To test this generalization in the present case, 8.2 Gm. of Salt *C* was subjected to barium hypoiodite oxidation (9) and 6 Gm. of white dibasic Barium Salt *D* was recovered. This salt no longer reduced Fehling's solution. The free aldehyde had been oxidized. The results of the analysis of Salt *D* are given in Table II. They show that it still contains a uronic acid confirming the above generalization.

Identification of L-Arabinose and D-Galactose.—Pentosan determinations and tests for pentoses and

TABLE II.—ANALYSIS OF BARIUM SALTS FROM HYDROLYSIS OF HONEY LOCUST GUM[a]

	Salt A	Salt B	Salt C	Salt D
Barium, %	9.21	15.68	16.10	27.14
Carbon dioxide, %	5.90	10.05	10.29	8.74
Uronic acid, %	25.75	43.85	44.90	38.13
L-Araban, %	44.10	12.25	8.61	0.00
D-Galactan, %	21.04	28.22	30.39	34.73
Araban units per uronic acid	2.49	0.41	0.28	0.00
Galactan units per uronic acid	0.97	0.76	0.80	0.97
Equivalent weight	745.00	438.00	427.00	503.00
$[\alpha]_D^{25}$	−18.40°	+45.36°	+34.20°	+29.43°

[a] Salt *A* is from gum heated for 1 hour at 65° in 2% sulfuric acid. Salt *B* is from gum heated for 3 hours at 80° in 4% sulfuric acid. Salt *C* is from Salt *B* heated for 2 hours at 100° in 4% sulfuric acid. Salt *D* is from Salt *C* oxidized to dibasic acid by barium hypoiodite.

methyl pentoses showed the presence of large amounts of a pentose and the absence of a methyl pentose (2, 7). During long heating of a polyuronide in acid solution some of the uronic acid is destroyed and the sugars combined with it are liberated. Hence, the sugar solutions obtained during the various acid hydrolyses of the gum and its salts would contain some of all the sugars present in it. None of any of the sugars or sugar syrups obtained during the many hydrolyses of the gum and its salts showed any sign of fermentation when treated with ordinary baker's yeast though control experiments were fermented. The sugars obtained by hydrolysis of the gum at 65° and 80° were crystallized from glacial acetic acid and showed $[\alpha]_D^{25}$ from +88° to +98°. When they were treated with alpha benzyl phenyl hydrazine they gave the alpha benzyl phenyl hydrazone of L-arabinose melting at 174°. This establishes the presence of L-arabinose in the gum and proves that it is the first sugar to be liberated. The ease of hydrolysis of this sugar suggests that it is present in the furanose form.

Sugars obtained from the hydrolysis of Salt *B* and at a number of other times during the investigation, when recrystallized from glacial acetic acid, showed $[\alpha]_D^{25}$ = +80° and on oxidation with nitric acid of 1.15 specific gravity gave mucic acid melting at 210–213°. This establishes the presence of D-galactose in the gum. When some of the aldobionic acid was heated with 4% sulfuric acid and the sugar was isolated and oxidized with nitric acid, mucic acid, melting at 212°, was obtained. This proves that D-galactose is attached directly to the uronic acid.

No indication of the presence of the gum of any other sugars than L-arabinose and D-galactose was met at any time.

Identification of D-Glucuronic Acid.—When aldobionic acids containing D-galacturonic acid are heated with hydrobromic acid and bromine as described by Anderson and Crowder (10) mucic acid, melting at 210–212° is obtained. When this test was applied to some Salt *C*, no mucic acid was obtained. Evidently D-galacturonic acid was not present in the aldobionic acid. The presence of D-galactose and the absence of D-glucose had already been established.

The method described by Anderson and Harris for establishing the presence of D-glucuronic acid in silk oak gum (11) was not applied. A total of 9 Gm. of the aldobionic acid was oxidized in an open dish on a bath of boiling water, with constant stirring, with 100 cc. of 20% nitric acid. After removal of the insoluble mucic acid formed from the D-galactose, D-saccharic acid was isolated from the oxidation mixture as potassium acid D-saccharic, with $[\alpha]_D^{25}$ = +8.2°. The potassium acid D-saccharate was converted to silver saccharate which, on analysis, gave 50.24% silver. Since potassium acid D-saccharate has $[\alpha]_D^{25}$ = +7.5° and silver saccharate has 50.91% silver, these data establish the presence of D-glucuronic acid in the gum

A spectroscopic analysis of the ash of the gum showed the presence of large amounts of calcium and strontium and no more than traces of other metals.

SUMMARY

The gum from the honey locust tree, *Gleditschia triacanthos* (L), is a polyuronide consisting of the calcium and strontium salts of D-glucuronic acid attached to a chain of molecules of anhydro D-galactose and anhydro L-arabinose. Analysis of the purified gum showed the presence on the average of 3.15 units of L-arabinose and 1.87 units of D-galactose to each unit of D-glucuronic acid. On mild hydrolysis with dilute sulfuric acid most of the L-arabinose and some of the D-galactose are liberated. More vigorous hydrolysis liberates all of the L-arabinose but leaves an aldobionic acid composed of D-glucuronic acid attached through its aldehyde group to one molecule of D-galactose. The gum is a pathological product and is found only on injured or diseased trees.

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2-Dibenzofuransulfonamides*

By THEODORE I. BIEBER†

The synthesis of 2-dibenzofuransulfonamide and of a number of its N-substituted derivatives is reported. These substances are readily accessible from dibenzofuran.

ALTHOUGH 2-dibenzofuransulfonic acid is readily prepared by sulfonation of dibenzofuran (1, 2), no 2-dibenzofuransulfonamides are known. The synthesis of 3-dibenzofuransulfonamide has, however, been reported (3). Since polycyclic sulfonamides may be pharmaceutically interesting, the synthesis of several representative 2-dibenzofuransulfonamides (listed in Table I) has now been carried out by permitting ammonia or the appropriate amine to react with 2-dibenzofuransulfonyl chloride. The latter, in turn, was conveniently obtained by the rapid interaction of free 2-dibenzofuransulfonic acid with phosphorus pentachloride in the presence of phosphorus oxychloride. A previous preparation of 2-dibenzofuransulfonyl chloride involved treatment of sodium 2-dibenzofuransulfonate with phosphorus oxychloride at 165–180° for a period of six hours (2). The direct chlorosulfonation of dibenzofuran to 2-dibenzofuransulfonyl chloride is largely vitiated by the ease with which dibenzofuran is disulfonated when allowed to react with a twofold molar quantity of chlorosulfonic acid. The introduction of a chlorosulfonyl group generally requires at least two moles of chlorosulfonic acid per mole of aromatic reactant.

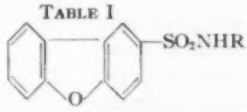
$$ArH + 2ClSO_3H \rightarrow ArSO_2Cl + H_2SO_4 + HCl$$

EXPERIMENTAL

2-Dibenzofuransulfonyl Chloride.—Dibenzofuran was converted to 2-dibenzofuransulfonic acid by means of concentrated sulfuric acid (1). When a mixture consisting of 20.0 Gm. of the anhydrous sulfonic acid, 32.5 Gm. of phosphorus pentachloride, and 45 cc. of phosphorus oxychloride was warmed, a vigorous reaction occurred with evolution of hydrogen chloride. The reaction mixture was refluxed for a few minutes, ice-cooled, and poured on 700 Gm. of ice. The lower layer of phosphorus oxychloride, in which the sulfonyl chloride was dissolved, was attacked only very slowly by the ice water on stirring. However, when the interface was left undisturbed for awhile so that the heat evolved by the slow hydrolysis of phosphorus oxychloride was permitted to produce a local temperature rise at the interface, then the hydrolysis quite suddenly became rapid and much heat was generated. At the first sign of this rapid hydrolysis the mixture was vigorously stirred and more ice was added if necessary. The 2-dibenzofuransulfonyl chloride precipitated as a white solid; it weighed 18.3 Gm. (85% crude yield) after being dried *in vacuo* and was used without purification for the preparation of the sulfonamides.

2-Dibenzofuransulfonamides.—The sulfonamides with R = H, CH_3, and C_2H_5 resulted from the reaction of 2-dibenzofuransulfonyl chloride with excess quantities of aqueous 33% ammonia, 25% methylamine, and 33% ethylamine. The sulfonamides were filtered at 60–70° to avoid possible contamination by ammonium or amine 2-dibenzofuransulfonates, which are of low water solubility (4). The yields were almost quantitative. For the recrystallization 75% ethanol was used when R was H, 60% ethanol when R was CH_3 or C_2H_5. 2-Dibenzofuransulfonanilide was obtained by adding the sulfonyl chloride to an excess of aniline and

TABLE I



		Analysis, %[a]								
		Carbon		Hydrogen		Nitrogen[b]		Sulfur[c]		M. P., °C.[d]
R	Formula	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found	
H	$C_{12}H_9NO_3S$	58.29	58.41	3.67	3.41	5.67	5.50	12.97	13.21	236–7
CH_3	$C_{13}H_{11}NO_3S$	59.75	59.98	4.25	4.20	5.36	5.48	12.27	12.00	133
C_2H_5	$C_{14}H_{13}NO_3S$	61.07	61.21	4.76	4.55	5.09	5.10	11.64	11.93	148.5
C_6H_5	$C_{18}H_{13}NO_3S$	66.86	66.79	4.05	4.07	4.33	4.51	9.91	9.95	180.5
2-pyridyl	$C_{17}H_{12}N_2O_3S$	62.95	63.00	3.73	3.86	8.64	8.57	9.88	9.55	242–3

[a] Performed by W. Manser, Zurich, Switzerland.
[b] By the micro-Dumas method.
[c] By the method of W. Zimmermann, *cf.* Grant, J., "Quantitative Organic Microanalysis Based on the Methods of F. Pregl," 5th English Edition, J. & A. Churchill Ltd., London, 1951, pp. 132–137.
[d] Uncorrected.

* Received March 30, 1953, from the Department of Chemistry, Coe College, Cedar Rapids, Iowa.
The help of Orville L. Kolsto, David C. Priest, and Gloria J. Rasch, all of whom were seniors majoring in chemistry during the academic year 1951–1952, is gratefully acknowledged.
† Correspondence concerning this article should be addressed to the author at 80-40 Lefferts Blvd., Kew Gardens 15, N. Y.

briefly heating to 100°. Dilute hydrochloric acid was then added and the sulfonamide was filtered (yield 93%). It was recrystallized from 70% ethanol. N-(2′-pyridyl)-2-dibenzofuransulfonamide was formed by the reaction of 2-dibenzofuransulfonyl chloride with 2-aminopyridine in pyridine solution. The product precipitated on dilution of the reaction mixture with water and was recrystallized from 75% ethanol.

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The Liquid-Liquid Countercurrent Separation of a Menthol-Menthyl Acetate Mixture*

By J. V. SWINTOSKY,† R. KURAMOTO,‡ and T. HIGUCHI

As a prelude to the use of a continuous multistage liquid-liquid countercurrent extractor for fractionation of volatile oils, it seemed desirable to employ a simple funnel assembly in making initial investigations on the applicability of the general countercurrent distribution method to these systems. The results of such a study, as applied to separation of a mixture of menthol and menthyl acetate, are presented in this paper.

Up to the present time, studies on the separation, isolation, and purification of volatile oil components have utilized mainly the principles of fractional distillation and crystallization. These methods have serious limitations particularly when the boiling points of individual components are very close, when the compounds are thermolabile, or when the compounds are liquid at room temperature and not readily amenable to purification by crystallization. Countercurrent distribution is free of these limitations since separation of compounds by this technique requires only a rational choice of immiscible solvents so that the partition coefficients of the respective compounds are reasonably different.

The theory and application of this type of separation are well known and are described in a number of conveniently accessible sources (1, 2).

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† Present address: Smith, Kline and French Laboratories, Philadelphia, Pa.
‡ This paper is based in part on a thesis submitted by Roy Kuramoto to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Master of Science.

By a derivation similar to that of Craig (1), an equation can be derived for calculating the fraction, $T_{n,r}$, of substance present in the rth stage for n transfers in a countercurrent distribution assembly:

$$T_{n,r} = \frac{n!}{r!(n-r)!} X^{n-r} Y^{r} \qquad (1)$$

where,

$$X = \frac{KR}{KR+1}$$

$$Y = \frac{1}{KR+1}$$

and where,

K = partition coefficient, and

$R = \dfrac{\text{volume of upper phase}}{\text{volume of lower phase}}$

The data which follow describe the agreement between experimental results and the theoretical predictions of Eq. 1, on the system under study.

EXPERIMENTAL AND RESULTS

It can be shown that the most efficient separation of two compounds by countercurrent distribution between two immiscible liquid phases of equal volumes is effected when the K value of the one compound is equal to the reciprocal of the other (1). The composition of the methanol-water phase which which would satisfy this condition when partitioned against petroleum ether (Skellysolve-*A*) is illustrated in the data of Fig. 1. Thus a methanol-water composition of 90.5/9.5 (v/v) was utilized for this study.

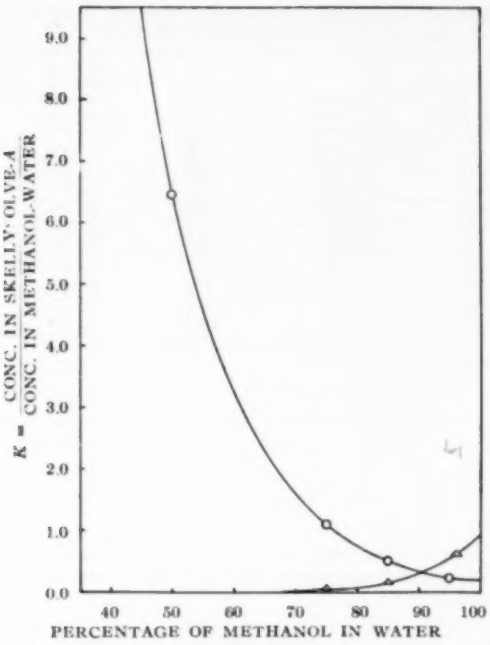

Fig. 1.—Partition coefficients for menthol and menthyl acetate between Skellysolve-*A* and methanol-in-water system. Δ, $1/K$ for menthyl acetate; O, menthol; temp. 25° C.

For the countercurrent fractionation of the menthol-menthyl acetate mixture, 100 ml. petroleum ether was placed in each of five separatory funnels numbered 0, 1, 2, 3, and 4. Accurately weighed samples, containing approximately 2 Gm. each of menthol and menthyl acetate, were dissolved in 100 ml. methanol water, and this solution placed in funnel 0. The contents of the funnel were shaken and, upon equilibration of the solute between the two phases, the lower liquid layer was transferred to funnel 1. One hundred milliters of methanol water was then added to funnel 0. This process was continued until four transfers were made. This method of fractionation was relatively rapid; and the entire transfers were conducted in less than one-half hour. The amounts of menthol and menthyl acetate contained in each liquid phase of each funnel were then determined essentially according to described methods (3, 4).

Before menthol could be determined by the usual acetylation method, it was necessary that the sample first be freed of methyl alcohol. This was accomplished for the hydroalcoholic samples by further dilution with water, addition of lithium chloride for its salting out effect and, finally, fourfold extraction with a 50-50 mixture of petroleum ether and diethyl ether. The combined extract was washed with water and dried over calcium chloride before analysis for menthol (3). The petroleum ether samples were simply washed with water and dried prior to analysis (3).

A comparison of experimental values and those calculated on the basis of Eq. 1 for this fractionation process is made in Table 1 and Fig. 2. The agree-

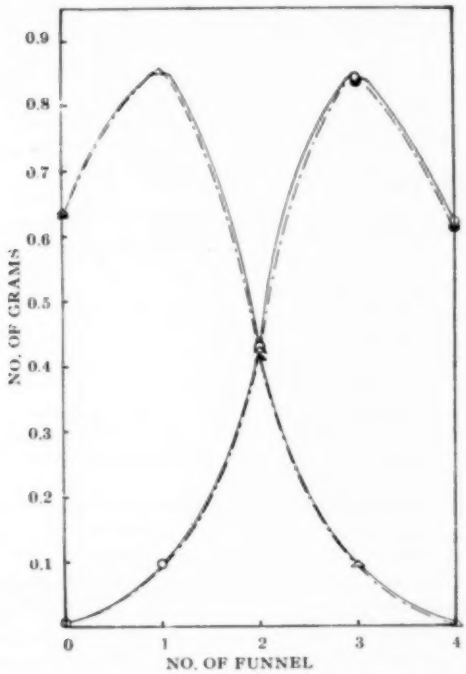

Fig. 2.—Countercurrent distribution curve using a Skellysolve-*A*/90.5% methanol-in-water system. Δ, menthyl acetate; O, menthol; —, calculated values; - - -, experimental values.

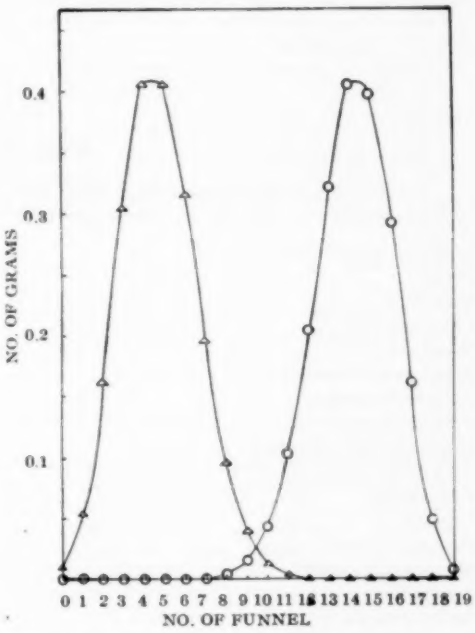

Fig. 3.—Countercurrent distribution curve from calculated values when using a Skellysolve-*A*/90.5% methanol-in-water system. Δ, menthyl acetate; O, menthol; 2.0-gram sample.

TABLE I.—FIVE-FUNNEL COUNTERCURRENT DISTRIBUTION OF MENTHOL-MENTHYL ACETATE MIXTURE BETWEEN THE SOLVENT PAIRS, PETROLEUM ETHER AND 90.5% METHANOL IN WATER[a]

	Funnel No.	Gm. in P. E., Experimental	Gm. in Methanol-H_2O, Experimental	Total Gm. per Funnel, Experimental	Total Gm. per Funnel, Calculated	Per Cent Purity of Compound for Funnel, Calculated
Menthol	0	0.000	0.006	0.006	0.008	1.24
	1	0.022	0.074	0.096	0.098	10.33
	2	0.109	0.318	0.427	0.431	50.35
	3	0.206	0.632	0.838	0.845	89.89
	4	0.156	0.456	0.612	0.621	98.73
Menthyl acetate	0	0.476	0.158	0.634	0.637	98.76
	1	0.635	0.214	0.849	0.851	89.67
	2	0.309	0.103	0.412	0.425	49.65
	3	0.072	0.024	0.096	0.095	10.11
	4	0.006	0.000	0.006	0.008	1.27

[a] Extractions were carried out at 25° under following conditions:
Weight of menthol = 2.0033 Gm.
Weight of menthyl acetate = 2.0163 Gm.
Volume of petroleum ether in each funnel = 100 ml.
Volume of methanol water in each funnel = 100 ml.

$$K \text{ for menthol} = \frac{\text{concentration in petroleum ether}}{\text{concentration in methanol water}} = 0.34$$

$$K \text{ for menthyl acetate} = \frac{1}{0.34}$$

ment is excellent, being within experimental error. Though five stages are insufficient to effect complete separation of the solute pair under the conditions of this experiment, it is apparent from the data that a considerable degree of separation results from only four transfers and that with an assembly possessing more stages, using the same solute-solvent system, higher predictable efficiencies of separation could be attained. This is illustrated in Fig. 3 where the same experimental conditions applied to the five-stage extraction assembly are simulated in a 20-stage extractor. The calculated values indicate that with complete recovery of the solute pair at least a 99% separation of the two components can be effected.

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WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Illinois, seeks information on sources of supply of the following chemicals:

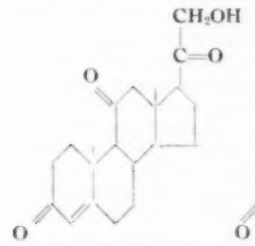
Purpurin-3-carboxylic acid
Zylan
Mannan
l-Limonene
Coniferin
Methanetrisulfonic acid
2,1,3,5-Tetrazole
β-Nitroethanol
Benzylpyridinium iodide
2,5-Diphenyloxazole
Zymosterol
Urobilin
Syringealdehyde
Sphingosine sulfate
Spermidine
Serine phosphate
Pyocyanine
Peroxidase
Ornithine phosphate
Canavanine sulfate
Pyridine-3,5-dicarboxylic acid
2,4,6-Trimethylphenol
Ethyl glyoxylate
β-Methylcrotonic acid
Calcium allylarsonate
Tetramethyluric acid
Glycyl-L-asparagine
Thymidine
Threonine phosphate
3-Nitrocarbazole
Capsaicine
Carnitine
Cephaelin
Agmantine
Absinthin
Ethanedithiol-1,2
l-β-Hydroxybutyric acid
4-Hydroxyproline
7-Methylguanine
1-Methyluric acid

The Chemical Assay of Adrenal Cortex Extracts*

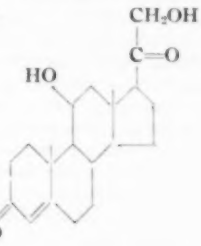
By DANIEL BANES

Milligram quantities of 11-dehydrocorticosterone, corticosterone, 17-hydroxycorticosterone and cortisone are separated from adrenal cortex extracts by partition chromatography and determined colorimetrically. Calculated glycogenic potencies show a close correlation with values obtained by bioassay.

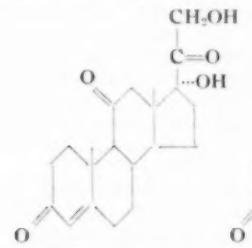
COMMERCIAL PREPARATIONS of adrenal cortex extracts are usually assayed by measuring their ability to promote the deposition of liver glycogen in adrenalectomized animals (1). Corticosteroids with an oxygen function at C 11 are the active substances in the test (2), and crystalline 11-dehydrocorticosterone (Kendall's Compound *A*), corticosterone (*B*), 17-hydroxy-11-dehydrocorticosterone (*E*), or 17-hydroxycorticosterone (*F*) can be employed as standard. However, the relative potencies of the steroids depend upon the modification of the procedure used (3–5), and values obtained for a given extract may vary significantly from laboratory to laboratory (5). An accurate chemical method for the determination of each of the active steroids in adrenal cortex extracts would provide an independent check for the bioassay method.



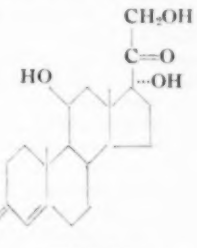
11-Dehydrocorticosterone (Kendall's Compound *A*)



Corticosterone (Kendall's Compound *B*)



17-Hydroxy-11-dehydrocorticosterone (Kendall's Compound *E*)



17-Hydroxycorticosterone (Kendall's Compound *F*)

Burton, Zaffaroni, and Keutmann (6, 7) have published the details of a chromatographic procedure for the separation of pure corticosteroids on paper, and Bush (8) has analyzed natural extracts by similar means. But the quantities of the materials isolated in such a chromatogram (10–50 micrograms of the pure substances) do not permit their examination for identity and purity as well as their estimation. The separation and quantitative recovery of milligram quantities of the active 11-oxygenated corticosteroids was accomplished by adapting the solvent systems of Burton, Zaffaroni, and Keutmann (7) to liquid-liquid partition chromatography on Celite.

EXPERIMENTAL

Materials.—Solvents.—(*a*) Benzene, c. p., redistilled in all-glass apparatus. (*b*) Formamide, 99%, manufactured by Matheson, Joliet, Illinois. (*c*) Isooctane, 99% (Pure Grade) produced by Phillips Petroleum Company, Bartlesville, Oklahoma.

Crystalline corticosteroids, used as supplied.[1]

Celite 545, manufactured by the Johns-Manville Company.

Chromatographic tube, prepared from a 200 x 25 mm. test tube. A small pledget of glass wool was inserted at the juncture of the tube and the stem, with a wad of purified cotton above it. The tube was fitted with a packing rod, prepared by flattening the end of a glass rod, about 300 mm. in length, to a circular head 20–23 mm. in diameter. A long-stemmed, stoppered 500-ml. separator served as reservoir for maintaining a constant volume of liquid in the tube.

Chromatographic Procedure.—Benzene (about 450 ml.) was mixed with 10.0 ml. of formamide and 10.0 ml. of water and the mixture was shaken vigorously several minutes. The layers were separated and each was filtered through paper into suitable beakers. Celite (8 Gm.) was mixed with the mobile solvent and 4.0 ml. of the polar solution was added, drop by drop with vigorous stirring, to produce a uniform solid phase. A portion of the solid was transferred to the chromatographic tube under liquid and pressed down firmly and evenly by means of the packing rod. The remainder of the stationary phase was similarly transferred in four or five portions, yielding a column 6–8 cm. in height with a flow-rate of 2–4 ml. of eluent per minute under a liquid head of about 6 cm. The mobile solvent was again filtered through paper and retained for chromatogram development.

Supernatant liquid was permitted to drain from the column and a prepared solution of corticosteroids in about 5 ml. of benzene was transferred to the tube

* Received June 12, 1953, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education and Welfare, Washington, D. C.

[1] The author is indebted to the following for gifts of crystalline corticosteroids: Merck and Co., Rahway, N. J., Syntex, S. A., Mexico City, D. F., and Upjohn and Co., Kalamazoo, Mich.

by pipette. The eluate was collected in a 100-ml. graduated cylinder. When the level of the liquid just reached the top of the column, the walls of the tube were washed with a 5-ml. portion of benzene delivered from the original container by means of the pipette. Container, pipette, and tube were then rinsed with three further 5-ml. portions of benzene in succession. About 30 ml. of mobile solvent was added to the tube, the reservoir was connected, and the chromatogram was developed. 11-Dehydrocorticosterone and corticosterone were eluted in the 70-ml. forerun. 17-Hydroxy-11-dehydrocorticosterone appeared in the next 100-ml. fraction and 17-hydroxycorticosterone in the following 230-ml. fraction.

Corticosterone and 11-dehydrocorticosterone were separated on a second column, employing (3 + 2) benzene + isooctane as mobile solvent and (4 + 1) formamide + water adsorbed on Celite as immobile phase; 300 ml. of benzene and 200 ml. of isooctane were shaken vigorously with 20.0 ml. of formamide and 5.0 ml. of water, and the layers were separated. The column was prepared as described above, beginning with the words "Celite (8 Gm.) was mixed...." The corticosteroids were dissolved in 3 ml. of benzene, and mixed with 2 ml. of isooctane. This solution was chromatographed in the prescribed manner. After a forerun of 70 ml., 11-dehydrocorticosterone was obtained in the next 80-ml. fraction and corticosterone in the following 160-ml. fraction.

DISCUSSION ON CHROMATOGRAPHY

The order in which the corticosteroids were eluted was the same as that found by Burton, Zaffaroni, and Keutmann (7), and confirmed by Bush (8) and by Grundy, Simpson, and Tait (9). Desoxycorticosterone preceded 11-dehydrocorticosterone and appeared in the forerun of the second column, while 17-hydroxydesoxycorticosterone was eluted with corticosterone. The chromatograms were developed at temperatures of 25–28°. Under other conditions, the partition properties of the pure corticosteroids in the solvent systems used should be ascertained before proceeding with the analysis of mixtures. Eight chromatograms (four samples) were completed in six hours easily, including the preparation of the columns, but they required the constant attention of the operator.

Typical results obtained in the chromatographic recovery of crystalline corticosteroids are shown in Table I. All recoveries were at least 93% of the added quantities, based upon the colorimetric tests described below.

Partition chromatography of the adrenal cortex extracts derived from commercial samples yielded fractions containing the substances desired, but they were frequently contaminated with other corticoids. The determination of 17-hydroxy-11-dehydrocorticosterone by the tetrazolium test described by Mader and Buck (10) yielded data only slightly higher than those obtained by the more specific phenylhydrazine procedure of Porter and Silber (11). There was also fair agreement in the estimations of 11-dehydrocorticosterone by ultraviolet spectrophotometry and by the tetrazolium test. But analysis of several corticosterone and 17-hydroxycorticosterone fractions showed the presence of interfering substances in large proportions. The infrared spectra of these fractions exhibited marked deviations from the spectra of the pure corticosteroids. In general, determinations based upon the tetrazolium test were much higher than those based upon ultraviolet spectrophotometry, indicating that the bulk of the concomitant steroids lacked the conjugated ketone function in Ring *A*.

It was hoped that a more specific test for the 11-hydroxycorticosteroids would permit their determination in the presence of these impurities. Treatment of 17-hydroxycorticosterone (10–100 μg.) with a 20% solution of concentrated sulfuric acid in glacial acetic acid (4.0 ml.) at 100° for one hour yielded a series of yellow-orange colors which obeyed Beer's Law at 450 mμ, the wave length of maximum absorption. Corticosterone behaved similarly, whereas 11-dehydrocorticosterone and 17-hydroxy-11-dehydrocorticosterone produced practically no color. However, the extraneous substances interfered in this test, also, and it was obvious that purification of the crude extracts before chromatography was necessary for accurate analyses.

In his pioneer work on the isolation of crystalline corticosteroids, Reichstein (12) found that ketones of the cholestenone type could be separated from less reactive ketones by the use of Girard's Reagent *T* under mild conditions. Experimentation with Reichstein's procedure showed that when milligram quantities of corticosterone or 17-hydroxycorticosterone were dissolved in 2 ml. of methanol containing 0.2 ml. of acetic acid and a large excess of Girard's Reagent *T*, the chloroform insoluble derivatives were formed quantitatively in two hours at room temperature. The corticosteroids were then regenerated by hydrolysis of the Girard derivatives at *p*H 1 for one hour. Extraction with chloroform, and estimation by means of the sulfuric-acetic acid test, led to recoveries of 95–98%.

Several mixtures of corticosteroids were subjected to treatment with Girard's Reagent *T*, followed by chromatographic partition. Results of these experiments indicated a high degree of accuracy for the extended procedure (Table II). Recoveries of 300–500 microgram quantities of corticosteroids added to a commercial product (sample 9) were particularly gratifying.

TABLE I.—RECOVERY OF PURE CORTICOSTEROIDS BY PARTITION CHROMATOGRAPHY

Sample No.	*A* Added, Mg.	*A* Recovered, Mg.	*A* %	*B* Added, Mg.	*B* Recovered, Mg.	*B* %	*E* Added, Mg.	*E* Recovered, Mg.	*E* %	*F* Added, Mg.	*F* Recovered, Mg.	*F* %
1	..	..	..	..	..	..	1.00	0.98	98	..	..	..
2	..	..	..	..	..	..	..	..	..	2.00	1.92	96
3	1.00	0.98	98	..	..	..	..	..	..	..	..	..
4	..	..	..	1.00	0.96	96	..	..	..	..	..	..
5	0.84	0.80	95	1.12	1.14	102	1.00	0.93	93	1.92	1.83	95

TABLE II.—RECOVERIES OF CORTICOSTEROIDS AFTER GIRARD REACTION AND CHROMATOGRAPHY

Sample No.	A			B			E			F		
	Added, Mg.	Recovered, Mg.	%	Added, Mg.	Recovered, Mg.	%	Added, Mg.	Recovered, Mg.	%	Added, Mg.	Recovered, Mg.	%
6	..	..	..	0.96	0.94	98	1.00	0.99	99	2.00	1.81	91
7	0.80	0.80	100	0.96	0.98	102	1.00	0.89	89	2.00	1.84	92
8	0.80	0.75	94	1.12	1.05	94	1.00	0.93	93	1.92	1.73	92
9[a]	0.40	0.40	100	0.32	0.30	93	0.50	0.46	92	0.40	0.37	92

[a] Pure steroids added to aliquot of Sample 14, and calculated by difference.

PROPOSED ASSAY PROCEDURE

Materials.—*Solvents*—Chloroform, methanol, alcohol, isooctane, acetic acid, and sulfuric acid of U. S. P. grade were used.

Girard's Reagent *T*.—The commercial product was recrystallized from absolute alcohol until the crystals were snow white and completely free of fishy odor. The dried solid was stored in tightly-covered brown bottles in a desiccator.

Solutions.—(*a*) Approximately molar sodium acetate solution; 82 Gm. of anhydrous sodium acetate (or 136 Gm. of the hydrated salt) was dissolved in 1 L. of water. (*b*) Hydrochloric Acid (3 *N*); 100 ml. of c. p. hydrochloric acid was diluted to 400 ml. with water.

Colorimetric Test Solutions.—(*a*) Tetrazolium chloride solution; 100 mg. of crystalline 2,3,5 triphenyltetrazolium chloride (as supplied by the Arapahoe Co. of Boulder, Colorado) was dissolved in a mixture of 16 ml. of alcohol and 4 ml. of formamide. The solution was prepared just prior to use. (*b*) Tetraethyl-ammonium hydroxide solution; 10 ml. of the 10% reagent in water (Eastman) was diluted to 100 ml. with alcohol. (*c*) Phenylhydrazine solution; 50 mg. of pure phenylhydrazine hydrochloride was dissolved in 30 ml. of water with the addition of 48 ml. of sulfuric acid, and the solution was cooled. The reagent was prepared just prior to use. (*d*) Sulfuric-acetic acid solution; 10 ml. of sulfuric acid was mixed with 40 ml. of acetic acid. The solution was prepared just prior to use.

Standard Solutions.—For each of the crystalline corticosteroids 25.0 mg. was dissolved in alcohol and diluted to 25.0 ml. with the solvent. The stock solution was diluted to yield solutions containing 25, 40, or 50 μg. of the substance per ml., as required.

Apparatus.—(*a*) Ultraviolet and visible spectrophotometer; a Beckmann Quartz Spectrophotometer, DU Model was used. (*b*) Infrared spectrophotometer; a Perkin-Elmer Double-beam Spectrophotometer, Model 21, Serial 147, was used in this investigation.

Preparation of Samples.—*Aqueous Solutions.*—An accurately measured aliquot of the aqueous solution of adrenal cortex extract containing 2–5 mg. equivalent of 17-hydroxycorticosterone was diluted with water to 75 ml. and extracted in a separator with seven 15-ml. portions of chloroform. Each chloroform extract was washed in a second separator with 10 ml. of water, and filtered through cotton. The combined chloroform extracts were evaporated to a small volume, transferred quantitatively to a 20-ml. beaker, and evaporated carefully to dryness.

Tablets.—A counted number of tablets containing adrenal cortex extract equivalent to 1–5 mg. equivalent of 17-hydroxycorticosterone were ground together, moistened with 15 ml. of water in a separator, and extracted with seven 40-ml. portions of chloroform. The chloroform extracts were washed, filtered, combined, and evaporated.

Oil Solutions.—An accurately measured aliquot of the oil solution containing adrenal cortex extract equivalent to 2–12 mg. of 17-hydroxycorticosterone was transferred to a separator and mixed with 50 ml. of isooctane. The mixture was extracted with ten 20-ml. portions of water faintly acidified (about 0.001 *N* HCl). Each aqueous extract was washed in a second separator with a further 50-ml. portion of isooctane, and removed to a 250-ml. separator. The combined aqueous layers were then treated like the samples under *Aqueous Solutions*.

Purification of Prepared Samples.—The residue obtained was dissolved in 2 ml. of methanol by warming on the steam bath, and the solution was cooled to room temperature. About 100 mg. of Girard's Reagent *T* and 0.2 ml. of acetic acid were added, and the mixture was permitted to remain at room temperature for two hours with occasional shaking. The solution was then mixed with 10 ml. of molar sodium acetate solution, and transferred to a separator with several 5-ml. portions of cold water. The aqueous mixture was extracted with seven 15-ml. portions of chloroform, and each chloroform extract was shaken with 10 ml. of wash water in a second separator. These chloroform extracts, containing nonketonic material and ketones relatively unreactive toward Girard's Reagent *T* were rejected. The aqueous solutions were combined, mixed with 5 ml. of 3 *N* hydrochloric acid, and allowed to stand one hour. Regenerated ketones were extracted with seven 15-ml. portions of chloroform. The extracts were washed, filtered through cotton, combined, and evaporated to dryness.

Analysis of Purified Extracts.—The ketonic residue was dissolved in 5 ml. of benzene and subjected to chromatographic partition according to the procedure described. Each significant fraction was evaporated to dryness and the residue was dissolved in a convenient quantity of alcohol. Approximate concentrations of the corticosteroid solutions were estimated by ultraviolet spectrophotometry. (A solution containing 40 μg. of pure corticosteroid per ml. possesses an absorbancy of about 1.7 at 240 mμ.) The individual substances were determined by means of the following procedures:

11-Dehydrocorticosterone (*A*).—Tetrazolium chloride solution (1.0 ml.) was added to 2.0 ml. of sample solution containing 30–60 μg. of 11-dehydrocorticosterone; 2.0 ml. of a standard solution containing 25 μg. of pure 11-dehydrocorticosterone in each ml., and a 2.0-ml. alcohol blank were similarly treated. To each mixture there was added 1.0 ml. of tetraethylammonium hydroxide solution and the ab-

sorbancies of the sample and the standard relative to the blank were measured at 480 mμ, reading at three-minute intervals until the maximum intensities were attained. The process was repeated with duplicate aliquots of the sample and standard, and the 11-dehydrocorticosterone content of the sample computed.

Corticosterone (B).—Duplicate aliquots of the sample solution containing 70–140 μg. of corticosterone were transferred to test tubes fitted with glass stoppers. A few carborundum chips were added and the solvent was evaporated by immersion in a steam bath until ebullition ceased. The warm tubes were placed in a vacuum desiccator for thirty minutes, to remove the last traces of alcohol. Duplicate tubes containing 100 μg. of pure, dry corticosterone were similarly prepared. To each tube there was added 4.0 ml. of sulfuric-acetic acid solution, and the tubes were stoppered loosely and heated in a steam bath for one hour. The corticosterone content of the sample was computed by comparing its absorbancy at 450 mμ, relative to a blank, with that of the standard. The tetrazolium test was employed as a check.

17-Hydroxy-11-dehydrocorticosterone (E).—Duplicate sample tubes containing 70–140 μg. of 17-hydroxy-11-dehydrocorticosterone and standards consisting of 100 μg. of the pure, dry steroid, were dissolved in 1.0 ml. of methanol. To each tube there was added 9.0 ml. of phenylhydrazine solution, the tubes were stoppered and shaken, and heated in a water bath at 60–65° for twenty minutes. The absorbancies of the cooled solutions were measured at 410 mμ, and the 17-hydroxy-11-dehydrocorticosterone content of the sample computed. The tetrazolium test was employed as a check.

17-Hydroxycorticosterone (F).—Fractions were treated as for corticosterone, except that samples contained 60–100 μg. and standards contained 80 μg. of the corticosteroid. Both the tetrazolium and phenylhydrazine tests were employed as checks.

Identification by Infrared Spectrophotometry.—After completion of the chemical analysis, the remainder of each sample solution was evaporated to a small volume, transferred to a 5-ml. weighing bottle, and evaporated to dryness. The residue was dissolved in two drops of pyridine, and the solution was transferred, by means of a dropper, to a platform, 8 x 15 mm., cut out of a sodium chloride crystal plate. The solvent was evaporated under vacuum, and the infrared absorption spectrum of the residual film was determined. Spectra for each of the standard corticosteroids were similarly prepared from 2 mg. of the pure substances.

DISCUSSION

The commercial adrenal cortex preparations of several manufacturers were assayed according to the proposed method. Results of assay are shown in Table III. The close agreement on duplicate aliquots in samples 11, 12, and 14 indicated a high degree of precision. Most of the residues were obtained as crystalline masses when evaporated from chloroform, but impurities from the column interfered with crystallographic identification. Their infrared spectra matched those of the standards, except that the 17-hydroxycorticosterone fractions of 12*a* and 12*b* (and of 19 and 20 below) still showed the presence of small quantities of an extraneous substance. As little as 0.8 mg. of material could be identified by infrared spectrophotometry.

The steroids found were assigned the relative glycogenic potencies reported by Haines (13) who followed the procedure of Pabst, Sheppard, and Kuizenga (5), and the total potencies were computed. These values ranged from 76–85% of the manufacturers' declared potencies for the solutions. In sample 14, about half of the computed potency was due to cortisone acetate (17-hydroxy-11-dehydrocorticosterone-21-acetate) added to the natural extract. This substance was eluted in the 11-dehydrocorticosterone and corticosterone fractions, and was identified by means of its characteristic infrared absorption spectrum in the 6 μ region. (The acetate crystallized out of pyridine, but deposited as a film out of chloroform.) Neither 11-dehydrocorticosterone nor corticosterone interfered in the phenylhydrazine test for cortisone acetate. Corticosterone was determined by means of the sulfuric-acetic acid test; 11-dehydrocorticosterone was estimated by difference in the tetrazolium test.

A further check of the proposed chemical method against a known bioassay procedure was made possible through the courtesy of the United States Pharmacopeia Panel on Adrenal Cortex Extracts. The Panel has been engaged in a study of extracts by a procedure similar to that of Pabst, Sheppard, and Kuizenga (5), and details of the study will be published elsewhere (14). Participating members —Parke, Davis and Co., Detroit, Mich., the Upjohn Co., Kalamazoo, Mich., and Wilson Laboratories, Chicago, Ill.—generously supplied portions of the

TABLE III.—ASSAY OF COMMERCIAL SAMPLES BY PROPOSED METHOD

Sample No.	Description of Sample	*A*, Mg.	*B*, Mg.	*E*, Mg.	*F*, Mg.	Total[a] as *F*, Mg.	Declared, %
10	Tablets; 100 contain equivalent of 1.25 mg. *F*	0.22	0.58	0.38	0.80	1.26	101
11*a*	Aqueous solution; 25 ml. equivalent	0.43	1.02	0.83	1.06	1.96	78
11*b*	to 5.00 mg. *E* (2.5 mg. *F*)[a]	0.42	1.02	0.82	1.01	1.91	76
12*a*	Aqueous solution; 25 ml. equivalent	0.62	0.94	0.93	0.92	1.90	76
12*b*	to 2.5 mg. *F*	0.63	0.91	0.91	1.01	1.98	79
13	Aqueous solution; 25 ml. equivalent to 6.25 mg. *F*	1.97	2.70	2.20	2.43	5.05	81
14*a*	Oil solution; 2 ml. equivalent to 2 mg.	0.26	0.22	0.33	0.53	0.85	85[b]
14*b*	*F*	0.23	0.25	0.32	0.53	0.84	85

[a] Based on the data of Haines (13). $A = \overline{0.28\ F}$; $B = 0.36\ F$; $E = 0.5\ F$.
[b] Sample contained cortisone acetate equivalent to 1.71 mg. *E* in 2 ml. aliquot. Total potency therefore is (1.71 x 1/2) + 0.85 = 1.71 mg. *F* or 85% of the declared potency.

collaborative samples for chemical assay. Results of analysis are shown in Table IV, together with the bioassay averages.

Values obtained by calculation from chemical determinations were 84–86% of the bioassay figures for the aqueous extracts. The ratios for the oil solutions were somewhat smaller. Tables I and II above indicate the normal losses of steroids treated according to the chemical assay procedure, and these losses would account for part of the discrepancies. A further deviation may be caused by the possible synergistic action of corticosteroids when administered together (3). Such a synergism would require extensive testing to establish the true glycogenic potencies of the active steroids in mixtures. However, the close correlation of the chemical values with the bioassay values shows that calculated potencies based upon the data of Haines (13) provide a reliable criterion for the glycogenic activity of adrenal cortex extracts.

It is recognized (2, 14, 15) that the liver-glycogen deposition test may not measure the most significant physiological activities of adrenal cortex extracts, but it represents the most convenient index for their biological standardization (14). The chemical assay method provides an index of similar validity with greater convenience and reproducibility. Furthermore, chemical analysis furnishes specific information about the constituents of adrenal cortex preparations, whereas bioassay methods measure only general physiological reactions.

SUMMARY

1. A method has been presented for the determination of 11-dehydrocorticosterone, corticosterone, 17-hydroxy-11-dehydrocorticosterone, and 17-hydroxycorticosterone in commercial preparations containing adrenal cortex extracts.

2. Milligram quantities of the steroids were separated by liquid-liquid partition chromatography, determined by chemical tests, and identified by infrared spectrophotometry.

3. Glycogenic potencies calculated from the concentrations of the substances in the extracts showed a close correlation with the values found by bioassay.

TABLE IV.—ASSAY OF COLLABORATIVE SAMPLES BY CHEMICAL METHOD AND BY BIOASSAY

Sample No.	Description of Sample	Chemical Analysis				Bioassay	
		A, Mg./Ml.	*B*, Mg./Ml.	*E*, Mg./Ml.	*F*, Mg./Ml.	Total[a] as *F*, Mg./Ml.	Total as *F*, Mg./Ml.
15*a*	Oil solution, 10 ml.	0.12	0.32	0.22	0.32	0.58	...
15*b*	Oil solution, 5 ml.	0.11	0.33	0.20	0.36	0.61	0.79
16	Oil solution, 25 ml.	0.07	0.18	0.12	0.18	0.33	0.45
17	Aqueous solution, 50 ml. (Sample 18 diluted to half strength)	0.021	0.039	0.032	0.054	0.090	0.104
18	Aqueous solution, 25 ml.	0.039	0.069	0.063	0.106	0.173	0.207
19	Aqueous solution, 50 ml.	0.076	0.062	0.066	0.032	0.109	0.129
20	Aqueous solution, 75 ml. (Sample 19 diluted to 0.6 strength)	0.048	0.036	0.038	0.018	0.063	0.075

[a] Based on the data of Haines (13): $A = 0.28\ F$; $B = 0.36\ F$; $E = 0.5\ F$.

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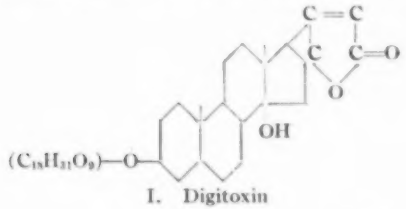
The Analysis of Digitoxin Tablets*

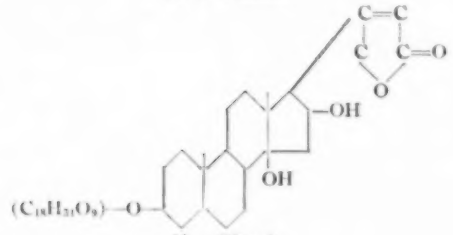
By DANIEL BANES and JONAS CAROL

A method is proposed for the isolation of pure digitoxin from digitoxin tablets by partition chromatography, and the determination of the glycoside by colorimetric procedures. The method permits the recovery and estimation of gitoxin and digitoxigenin in digitoxin tablets.

THE U. S. P. XIV monograph on digitoxin (1) defines the drug as "either pure digitoxin ($C_{41}H_{64}O_{13}$) or a mixture of cardioactive glycosides obtained from *Digitalis purpurea* . . . and consisting chiefly of digitoxin." The prescribed method of assay is based upon the reaction of trinitrophenol with the α-β unsaturated lactone ring of digitoxin (I) to produce an orange color. All steroids which possess the butenolide ring produce a similar color when treated with trinitrophenol (2, 3). Gitoxin (II), the chief concomitant glycoside derived from *Digitalis purpurea* (4), yields a trinitrophenol color about four-fifths as intense as that due to digitoxin (5).



I. Digitoxin



II. Gitoxin

Since U. S. P. Digitoxin Reference Standard itself may contain steroids less chromogenic than digitoxin (4, 6) and, since assayed powders are required to produce a trinitrophenol color only 95 per cent as intense as the color developed by this standard, samples containing 70 per cent digitoxin may be found to conform with all the requirements of the monograph.

Preparations containing digitoxin are also assayed officially by the use of the trinitrophenol test (7). In the case of digitoxin tablets, the powdered material is first extracted with chloroform in a micro-Soxhlet extractor, and the residue, after evaporation of the chloroform, is dissolved in alcohol for analysis. But some glycosides, including gitoxin, may not be recovered completely by such an extraction. Thus tablets comprised of the specified quantity of "digitoxin" as defined in the monograph on digitoxin may be found to contain less than the minimum quantity of "digitoxin" required by the assay rubric in the monograph on digitoxin tablets (90 per cent of the declared quantity). Samples 1–4 in Table I were assayed by the U. S. P. method and the residues were tested for the presence of unextracted glycosides. All of these residues showed evidence of incomplete extraction. On the other hand, several samples (Samples 5, 15, 16) contained tablet excipients soluble in chloroform which interfered with color absorption measurements in the trinitrophenol test and led to high assay values. Therefore it seemed desirable to investigate the utility of other colorimetric tests for the estimation of the cardioactive steroids in digitoxin tablets, and to devise procedures for the isolation, identification, and determination of these steroids.

Most of the methods proposed for the chemical estimation of the digitalis glycosides depend upon the reactivity of either the digitoxose moiety or the butenolide ring in the aglycone. Rowson (8) has re-examined several of these reactions, and has described appropriate conditions for their use. His modification of the Keller-Kiliani test for digitoxose, based upon the investigations of Soos (9) and of James, *et al.* (10), appeared to yield the most accurate results for the determination of the individual glycosides. The Raymond *m*-dinitrobenzene test for the butenolide ring (11), performed in weakly alkaline solution, as suggested by Hassall and Lippman (12), proved a convenient and reliable test for the cardioactive aglycones. The Windaus-Schwarte sulfuric acid test for gitoxigenin (13) was employed to check the gitoxin content of several samples analyzed by chromatographic partition.

Liquid-liquid partition chromatography has been utilized successfully for the quantitative analysis of complex steroid mixtures, such as urinary estrogens (14, 15, 16) and the hormones of the adrenal cortex (17). Stoll and his collaborators (18) have separated digitalis glycosides by chromatographic techniques, and they em-

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ployed adsorption chromatography for the large-scale separation of digitoxin from gitoxin (6). By the use of a formamide-water solution as immobile phase adsorbed on Celite, and a benzene-chloroform solution as mobile solvent, a chromatographic system has now been devised which permits the quantitative segregation of digitoxin, as well as the recovery of gitoxin and the detection of digitoxigenin in digitoxin tablets. The solubility differences of digitoxin and gitoxin in this solvent system were so marked that a sorting procedure based upon simple immiscible solvent extraction has been employed for the separation of the two glycosides in mixtures.

EXPERIMENTAL

Materials

Solvents.—(*a*) Benzene, c. p., was redistilled in an all-glass apparatus; (*b*) formamide, 99%, manufactured by Matheson, Joliet, Illinois; (*c*) chloroform, U. S. P. grade. The solvent, when employed in chromatographic development, was washed four times with an equal volume of water and filtered through paper immediately before use.

Solutions—(*a*) *Acidified sodium sulfate.*—Sodium sulfate, 100 Gm., was dissolved in water, mixed with 4 ml. of concentrated hydrochloric acid, and diluted with water to a liter. (*b*) *Keller-Kiliani reagent.*—Glacial acetic acid, 60 ml., was mixed with 5 ml. of concentrated sulfuric acid and 2 ml. of 5% ferric chloride solution. (*c*) *m-Dinitrobenzene reagent.*—Crystalline *m*-dinitrobenzene (Eastman), 2 Gm., was dissolved in 100 ml. of ethanol. (*d*) *Tetraethylammonium hydroxide solution.*—Tetraethylammonium hydroxide in water (Eastman), 5 ml. of 10% solution, was diluted to 50 ml. with ethanol. (*e*) *Windaus-Schwarte reagent.*—Ferric chloride, 0.2 ml. of 5% solution, was mixed with 250 ml. of concentrated sulfuric acid.

Chromatographic Tube.—The tube was prepared from a 200 × 25 mm. test tube. A small pledget of glass wool was inserted at the juncture of the tube and the stem, with a wad of purified cotton above it. The tube was fitted with a packing rod prepared by flattening the end of a glass rod, about 300 mm. in length, to a circular head 20–23 mm. in diameter. A long-stemmed, stoppered 500-ml. separator served as reservoir for maintaining a constant volume of liquid in the tube. Celite 545, manufactured by the Johns-Manville Company, was used as adsorbent for the immobile phase.

Spectrophotometer.—A Beckmann DU quartz spectrophotometer with 1 cm. Corex cells was used.

Crystalline Steroids.—U. S. P. Reference Standard Digitoxin, and samples of gitoxin and digoxin were used as supplied by the U. S. P. Committee of Revision. Digitoxigenin was prepared from digitoxin by the method of Stoll and Kreis (19). Chromatographically pure digitoxin was obtained as follows: 200 mg. of impure digitoxin was dissolved in about 10 ml. of hot chloroform, and transferred to a chromatographic column prepared similarly to that described below. The chromatogram was developed with benzene as eluent, and the first 30 ml. of eluate were rejected. Evaporation of the next 450 ml. of eluate yielded 120 mg. of crude digitoxin, essentially free of gitoxin, and 100 mg. of this material was recrystallized twice from 50% alcohol, yielding 62 mg. of white crystals. When 5 mg. of the substance was subjected to chromatographic analysis according to the methods described below, at least 99% appeared in the digitoxin fraction, with less than 0.8% in the gitoxin residue and less than 0.2% in the digitoxigenin forerun.

Proposed Method of Analysis

Preparation of the Sample.—A counted number of tablets were ground to a fine powder, and a quantity of powder equivalent to 5 mg. of digitoxin was mixed in a beaker with 15 ml. of methanol. The beaker was covered with a watch glass, and the mixture was carefully heated on the steam bath at the boiling point for twenty minutes, with frequent stirring. The cooled mixture was transferred to a separator with the aid of 150 ml. of acidified sodium sulfate solution and 50 ml. of chloroform. The mixture was shaken vigorously, and the chloroform extract was transferred to a second separator containing 15 ml. of wash water. After washing, the chloroform solution was filtered through cotton into a beaker. The extraction process was repeated with six 25-ml. portions of chloroform, and the combined chloroform solution was evaporated to 2–3 ml. The concentrated solution was then transferred to a small flask (a 50 × 15 mm. weighing bottle was found convenient) with the aid of a stream of chloroform. The beaker was rinsed with several small portions of the solvent and the rinsings were added to the weighing bottle. The combined solutions were then evaporated to dryness. The residue was moistened with 0.5 ml. of chloroform, the bottle was stoppered loosely, and the residue was dissolved by warming. The cooled solution was then mixed with 4.5 ml. of benzene for chromatographic separation.

Preparation of the Chromatographic Column.—Washed chloroform, 50 ml., was transferred to a 500-ml. volumetric flask and diluted to the mark with benzene. The solution was then shaken five minutes with 20.0 ml. of formamide and 10.0 ml. of water. Each of the two layers was filtered through a dry paper filter. Ten grams of celite was mixed with the chloroform-benzene solution and 5.0 ml. of the polar solution was added drop by drop with vigorous stirring to produce a uniform solid phase. A portion of the solid was transferred to the chromatographic tube under liquid and pressed down firmly and evenly by means of the packing rod. The remainder of the stationary phase was similarly transferred in four or five portions, yielding a column 6–9 cm. in height with a flow-rate of 2–4 ml. of eluent per minute under a liquid head of about 6 cm. The mobile solvent was again filtered through paper and retained for chromatogram development.

Chromatographic Procedure.—Supernatant liquid was permitted to drain from the column and the prepared solution of digitoxin extract in the weighing bottle was transferred to the tube with the aid of a stream of (1 + 9) chloroform-benzene solution. The eluate was collected in a 50-ml. graduated cylinder. When the level of the liquid just reached the top of the column, the walls of the tube were washed with a 5-ml. portion of the mobile solvent delivered from the weighing bottle. Bottle and tube were rinsed in a similar fashion with three further 5-ml.

portions of chloroform-benzene solution in succession. About 30 ml. of eluent was added to the tube, the reservoir was connected, and the chromatogram was developed. The first 45 ml. of eluate forerun was retained for the detection of digitoxigenin, while the next 250 ml. of eluate was collected as the digitoxin fraction. The column was then permitted to drain and the immobile phase was extruded into a beaker by means of an air jet applied to the stem of the tube. The moist solid was stirred with about 150 ml. of water and washed into a separator with several portions of water. After acidification with a few drops of hydrochloric acid, the mixture was extracted with four 50-ml. portions of chloroform. The chloroform extracts were washed, combined, and evaporated to yield the gitoxin-containing residue.

Colorimetric Determinations.—The digitoxin fraction was evaporated to dryness, dissolved in alcohol, and diluted to exactly 25 ml. with alcohol. Duplicate 2.0-ml. aliquots were transferred to test tubes and evaporated in a steam bath with the aid of carborundum boiling chips, until ebullition ceased. The tubes were then dried and cooled in a vacuum desiccator for thirty minutes. Duplicate tubes, each containing 400 μg. of digitoxin were similarly prepared from a standard solution containing 200 μg. of the purified glycoside per ml. Four milliliters of Keller-Kiliani reagent was added to each tube and they were shaken frequently to dissolve the solids. After twenty minutes the absorbancies of the blue-green colors were measured relative to the reagent blank at 590 mμ, and readings were recorded at two-minute intervals until the maximum values were attained. The digitoxin content of the sample was computed by comparison of its absorbancy with that of the standard.

In the test for the butenolide ring another set of duplicate 2.0-ml aliquots was measured out for both digitoxin sample and standard; 1.0 ml. of *m*-dinitrobenzene reagent was mixed with each aliquot, followed by 1.0 ml. of tetraethylammonium hydroxide solution. A 2.0-ml. alcohol blank was similarly treated. The solutions were mixed, and the absorbancies of the developing red-violet colors were measured at 560 mμ at two-minute intervals relative to the blank, until the maximum intensity was ascertained. The digitoxin content of the sample was then computed.

Digitoxigenin was detected by applying the Raymond and Keller-Kiliani tests to the residues obtained from 20-ml. portions of the forerun. A positive Raymond test coupled with a negative Keller-Kiliani test was accepted as evidence for the presence of the genin.

The gitoxin-containing residue was dissolved in 10 ml. of ethanol. Two-milliliter aliquots were tested by the two methods described and compared with a standard gitoxin solution containing 200 μg. per ml. The glycosides found were calculated as gitoxin. The glycoside produced a color almost as intense as that due to digitoxin in the Keller-Kiliani test, but only 0.3 as intense in the Raymond test. The Windaus-Schwarte test was applied to a sample of digitoxin powder (sample 12) to check its gitoxin content as follows: 20.0 ml. of Windaus-Schwarte reagent was added to 0.51 mg. of sample 12 and the mixture was shaken five minutes to achieve solution. A set of standards (comprised of 300 μg. of digitoxin, 300 μg. of digitoxin plus 150 μg. of gitoxin, 300 μg. of digitoxin plus 200 μg. of gitoxin, and 200 μg. of gitoxin) was similarly treated. The absorbancies of the developing red colors were then measured relative to the reagent blank at 530 mμ repeatedly during the next ten minutes. The gitoxin content of the sample was computed from the maximum absorbancies, by comparison with the appropriate mixed standards.

Each of the colors obtained in these three tests developed at a convenient rate (5–20 minutes), and proved stable at their maximum absorption long enough (3–10 minutes) to permit accurate measurements. All of the maximum colors generated by 100–500 μg. of the glycosides obeyed Beer's Law. The reagents described were stable, and yielded uniform colors over a period of several days.

Sorting Procedure.—A sample equivalent to 5 mg. of digitoxin was extracted as described under *Preparation of the Sample*, and the dried residue was heated on the steam bath with 25 ml. of chloroform for ten minutes. The cooled solution was washed into a clean, dry separator with 50 ml. of benzene, and extracted with four 25-ml. portions of a solution prepared by mixing 50 ml. of formamide with 50 ml. of the acidified sodium sulfate solution. The aqueous extracts were washed in succession in a second and third separator, each containing 25 ml. of (2 + 1) benzene + chloroform. The combined aqueous layers were diluted with 300 ml. of water and extracted with four 40-ml. portions of chloroform. The chloroform extracts were washed with water, combined, and examined for gitoxin. The benzene-chloroform solution in the first separator was shaken with 30 ml. of water, removed to a fourth separator containing 30 ml. of water, and the washing was repeated. The organic layer was then filtered through cotton into a beaker. The benzene-chloroform solutions, in the second and third separators were each similarly washed in the first and fourth separators and combined with the chief organic extract as the digitoxin-containing fraction. Glycosides were then determined colorimetrically according to the procedures described.

DISCUSSION

The proposed chromatographic procedure was applied to three known mixtures of gitoxin and purified digitoxin (samples 6–8), and to U. S. P. Digitoxin Reference Standard, while the complete method was employed for the analysis of two samples of tablets alleged to contain 0.2 mg. of the U. S. P. standard per tablet. Results of these analyses, presented in Table II, indicated the achievement of quantitative recoveries of digitoxin from mixtures and highly satisfactory assay values for the tablets. All analyses on material comprised of U. S. P. Digitoxin Reference Standard showed it to contain 2–3% of other glycosides as gitoxin. Stoll (6) and McChesney, *et al.* (4), reported similar findings on their samples of U. S. P. Digitoxin Reference Standard. Discrepancies between the Keller-Kiliani test and the Raymond test were no greater than 5% for any digitoxin fraction. Data reported here were based upon the Keller-Kiliani test.

Samples 1–5 (Table I), which failed to conform to the U. S. P. assay requirements for digitoxin tablets, were then analyzed by the proposed method. On

TABLE I.—COMPARISON OF U. S. P. ASSAY WITH PROPOSED METHOD

Sample	Declaration Digitoxin per Tablet, μg.	U. S. P. Assay: Digitoxin Found, μg.	U. S. P. Assay: Declared, %	Assay by Proposed Method: Digitoxin Found, μg.	Gitoxin Found, μg.	Total μg.	Declared, %
1	200	156	78	167	34	201	100
2	200	160	80	158	27	185	92
3	200	160	80	158	43	201	100
4	200	147	74	154	27	181	90
5	200	256	128	157	33	190	95

TABLE II.—ANALYSIS OF KNOWNS BY PROPOSED METHOD

Sample	Description	Digitoxin, Mg.	Gitoxin, Mg.	Total, Mg.	Recovery or Declared, %
6	5.00 mg. purified digitoxin plus 0.40 mg. gitoxin	5.15	0.34	5.49	101.7
7	5.00 mg. purified digitoxin plus 0.60 mg. gitoxin	4.95	0.63	5.58	99.6
8	5.00 mg. purified digitoxin plus 1.00 mg. gitoxin	4.82	1.32	6.14	102.3
9	5.00 mg. U. S. P. Digitoxin Reference Standard	4.93	0.11	5.04	100.8
10	25 starch tablets containing 5 mg. U. S. P. Digitoxin Reference Standard	4.65	0.13	4.78	95.6
11	25 bentonite tablets containing 5 mg. U. S. P. Digitoxin Reference Standard	4.94	0.14	5.08	101.6

TABLE III.—COMPARISON OF U. S. P. CHEMICAL AND BIOASSAYS WITH PROPOSED METHOD

Sample	Description	U. S. P. Assay[a] Declared, %	Bioassay[a] Declared, %	Assay by Proposed Method: Digitoxin	Gitoxin	Total	Declared, %
12	Commercial digitoxin powder	100	77; 80	62.8%	37.2%	100%	100
13	0.1 mg. digitoxin tablets	102	83	0.078	0.016	0.094	94
14	0.2 mg. digitoxin tablets	99	82	0.161	0.028	0.189	94
15	0.1 mg. digitoxin tablets	141	99; 101	0.077	0.017	0.094	94
16	0.2 mg. digitoxin tablets	190	101; 104	0.176	0.028	0.204	102

[a] Assays performed by Dr. H. A. Braun of the Division of Pharmacology, U. S. Food and Drug Administration.

the basis of the total glycosides recovered, all five samples were found to contain 90–100% of the declared quantity of digitoxin.

In a recent report on a series of twenty-eight commercial preparations containing digitoxin, Braun (20) has observed discrepancies between the data obtained by the U. S. P. chemical assay for digitoxin and by the U. S. P. bioassay for digitalis. Samples 12–16 in Table III represent five of the products studied by Dr. Braun. He had found that three of these samples complied with the requirements of the U. S. P. chemical test, but yielded low bioassay values; the others yielded satisfactory bioassay results, but exceeded the maximum limit of the U. S. P. monograph when assayed chemically. Analysis by the proposed method demonstrated that all of these samples contained 94–102% of the declared quantities of total cardioactive glycosides.

None of the commercial samples showed more than a trace (less than 0.5%) of digitoxigenin or other butenolide steroid in the chromatographic foreruns. Colorimetric examination of the gitoxin-containing residues by the Keller-Kiliani, Raymond, and Windaus-Schwarte tests indicated that they were composed chiefly, if not exclusively, of gitoxin. The behavior of digitoxin on the chromatographic column differed so markedly from that of gitoxin (and of digoxin) that a sorting procedure was devised for their separation by immiscible solvent extraction. Results obtained by applying this sorting procedure to four known mixtures of gitoxin and purified digitoxin and to four tablet samples previously analyzed by the chromatographic method are presented in Tables IV and V, respectively. The sorting procedure proved rapid and fairly accurate, but it did not segregate the primary substance, digitoxin, from other steroids and tablet excipients as efficiently as chromatography. A large quantity of digitoxin powder (800 mg. of Sample 12) was subjected to the sorting procedure. Gitoxin separated during the extractions, but the digitoxin content of the material soluble in benzene-chloroform rose from 63–92%, with small losses of the glycoside. A more efficient solvent system could perhaps serve to facilitate the purification of large masses of digitoxin.

The gitoxin content of Sample 12 was checked by the application of the Windaus-Schwarte test for gitoxigenin to the original powder, as previously described. By this independent test, also, gitoxin was found to comprise 37–38% of the total glycosides. The low bioassay value of Sample 12 yields the calculated toxicity for gitoxin as 40–45% of that for

TABLE IV.—ANALYSIS OF KNOWNS BY THE SORTING PROCEDURE

Sample	Digitoxin Added, Mg.	Digitoxin Recovered, Mg.	Gitoxin Added, Mg.	Gitoxin Recovered, Mg.	Total Added, Mg.	Total Recovered Mg.	Recovery, %
17	4.50	4.50	1.00	0.94	5.50	5.44	98.9
18	4.00	3.97	1.00	0.94	5.00	4.91	98.2
19	3.50	3.61	1.00	0.90	4.50	4.51	100.2
20	2.50	2.67	1.00	0.84	3.50	3.51	100.3

TABLE V.—COMPARISON OF CHROMATOGRAPHIC AND SORTING METHODS

Sample	Digitoxin Chromat., Mg./Tab.	Digitoxin Sorting, Mg./Tab.	Gitoxin Chromat., Mg./Tab.	Gitoxin Sorting, Mg./Tab.	Total Chromat., Mg./Tab.	Total Sorting, Mg./Tab.
10	0.186	0.182	0.005	0.007	0.191	0.189
11	0.198	0.194	0.006	0.005	0.204	0.199
15	0.161	0.165	0.028	0.030	0.189	0.195
16	0.176	0.180	0.028	0.024	0.204	0.204

U. S. P. Digitoxin Reference Standard in pigeons. Potencies previously reported ranged from 35–40% of digitoxin when frogs were used as test animals (21, 22), and from 35–83% in cats (21, 22, 4). But no data were encountered on the therapeutic efficacy of gitoxin, when administered orally to human beings. Therapeutic variations between tablets containing pure digitoxin and those containing powders with a high gitoxin content could be eliminated by modifying the official definition of digitoxin to include only the pure glycoside, with tests provided to limit the maximum quantity of other cardioactive steroids permitted.

SUMMARY

1. A method has been proposed for the analysis of mixtures containing digitoxin and related steroids.

2. The substances were separated by liquid-liquid partition chromatography, and determined colorimetrically by means of the Keller-Kiliani and Raymond reactions.

3. The method has been applied successfully to the assay of commercial digitoxin tablets.

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In Vivo Capacity of High Porosity Cation Exchange Resins*

By GUSTAV J. MARTIN, SIDNEY ALPERT, and MILLER J. SULLIVAN

A REDUCTION of the difficulties of cation exchange resin application in various clinical states which require sodium restriction is concerned with efforts to increase the *in vivo* capacity of such materials. One approach, the combined use of chelating agents with cation exchangers, has been reported by Martin, *et al.* (1), to increase markedly the *in vivo* capacity. It has been generally considered impossible to modify materially the intrinsic capacity of cation exchange resins and the resins reported in this communication do not exhibit a significantly increased *in vitro* capacity, but do manifest greatly augmented *in vivo* power. The modification in the form of resin lies in the increased porosity.

* Received May 21, 1953, from the Research Laboratories, The National Drug Co., Philadelphia, Pa.

TABLE I.—THE IN VIVO CAPACITY OF CATION EXCHANGE RESIN OF VARYING POROSITY[a]

	Natrinil, 5%	XE-64, 5%	Resin *A*, 5%	Resin *B*, 5%	Controls
Fecal sodium	...	168 (7)[b]	...	261 (7)	44 (21)
	100 (7)	...	183 (7)	207 (7)	34 (20)
	...	167 (7)	215 (7)	273 (7)	40 (21)
Urinary sodium	...	270 (7)	...	220 (7)	324 (21)
	265 (7)	...	238 (6)	208 (7)	338 (20)
	...	199 (7)	204 (7)	161 (7)	360 (21)
Fecal potassium	...	118 (7)	...	134 (7)	88 (21)
	108 (7)	...	109 (7)	108 (7)	60 (20)
	...	114 (7)	113 (7)	118 (7)	69 (21)
Urinary potassium	...	397 (7)	...	401 (7)	447 (21)
	486 (7)	...	361 (6)	391 (7)	472 (20)
	...	307 (7)	310 (7)	338 (7)	399 (21)
Fecal calcium	...	670 (7)	...	679 (7)	683 (21)
	613 (7)	...	703 (7)	659 (7)	960 (20)
	...	725 (7)	719 (7)	726 (7)	724 (21)
Urinary calcium	...	6 (7)	...	9 (7)	13 (21)
	6 (7)	...	9 (6)	8 (7)	12 (20)
	...	5 (7)	8 (7)	5 (7)	17 (21)
Fecal chloride	...	102 (7)	...	111 (7)	106 (21)
	102 (7)	...	97 (7)	115 (7)	84 (20)
	...	125 (7)	123 (7)	140 (7)	96 (21)
Urinary chloride	...	503 (7)	...	489 (7)	511 (21)
	482 (7)	...	513 (6)	476 (7)	537 (20)
	...	437 (7)	505 (7)	485 (7)	494 (21)

[a] All values Mg. per 100 Gm. of ingested food.
[b] Figures in parentheses refer to the number of pairs of rats.

EXPERIMENTAL

The experimental design paralleled in large measure that employed by McChesney and his associates (2–4) and Ch'en and Freeman (5). Natrinil, a commercial product, which is a purified carboxylic cation exchanger in a very finely divided state (90% through 200 mesh), 20% in the potassium cycle, and 80% in the hydrogen cycle, was used as a control form. In addition, three resin forms, Amberlite XE-64 and resins *A*[1] and *B*[1] were employed for test. These varied primarily in their porosity with pore size greater from Natrinil and XE-64 to resin *A* to resin *B*. All are carboxylic cation exchange resins. The diet and analytical methods employed have been fully described by Sullivan, *et al.* (6).

RESULTS AND DISCUSSION

From Table I, it is apparent that the carboxylic resins of greater porosity manifest increased *in vivo* capacities in the range of 100%. *In vitro*, the capacity of these resins is essentially similar, being approximately 10 meq. per dry gram; while *in vivo*, the approximate values are 1.0 for XE-64, 1.4 for Resin *A*, and 2.08 for Resin *B*. The result observed is therefore not a manifestation of increased total capacity as reflected in the *in vitro* values but rather the realization of a greater percentage of total capacity *in vivo*. For Amberlite XE-64, the percentage is 10%; for Resin *B*, close to 20% of maximal *in vitro* capacity is realized *in vivo*. Practical considerations would seem to preclude the development of a resin of greater porosity than sample B. The probability is therefore great that this resin possesses a near maximal *in vivo* capacity.

In trying to explain these findings, we offer as pure conjecture the possibility that greater porosity results in greater availability of carboxylic groups for exchange. In many instances with the smaller porosity resins, the pore ends are plugged by large organic molecules which effectively block the reactive groups deeper within the pore. When the pore reaches a critical size, comparatively few of the pores are so blocked with the result that availability is markedly increased. Diffusion rate alterations offer a second possible basis. Explanation of the greater selectivity *in vivo* of resins *A* and *B* for sodium is not immediately apparent but the results are clear-cut.

SUMMARY

1. Carboxylic cation exchange resins with greater porosity than those in general usage manifest *in vivo* markedly increased capacity.

2. Such increases in *in vivo* capacity are not reflected *in vitro*.

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[1] Obtained through the courtesy of Dr. James Winters, Rohm and Haas, Philadelphia, Pa.

Paper Chromatography of Choline and the Vitamins B_1, B_2, Niacin, and Niacinamide*

Preparation of Radioactive Choline Acetate and Study of Its Hydrolysis

BY AUBIN HEYNDRICKX†

A qualitative study was made by means of paper chromatography of these different compounds. A suitable solvent, *n*-propyl alcohol and hydrochloric acid, was used to separate them quickly. The study is devoted to the preparation and hydrolysis of radioactive choline acetate. Spot tests for the products are described.

VITAMIN MEDICAMENTS are frequently administered in complex forms. In order to control the preparations, it is of great importance to set up a selective determination which can be performed easily in a control laboratory. The water soluble vitamins have nearly the same solubilities in different solvents and at best separate only semiquantitatively. A suitable method for their identification is paper chromatography (1–3), which gives fast and reproducible results.

Choline acetate could not be determined directly by the method used for the vitamins. An explanation for this phenomenon is offered and a study of the decomposition of choline acetate is reported.

Color reactions and paper chromatograms on pantothenic acid derivatives have previously been reported (4). A quantitative determination for niacin and niacinamide was worked out by Wohlers (5).

Choline acetate plays a very active role in metabolism and physiology. In order to investigate the stability of the product, radioactive choline acetate was prepared and checked chromatographically on paper.

EXPERIMENTAL

Whatman filter paper No. 4 was used and cut in strips 4.3 cm. wide and 40 cm. long. The apparatus was set up for a downward migration technique, using a cylindrical jar[1] manufactured by the Fisher Scientific Company.

The paper sheets with the substance applied were hung overnight in an atmosphere of *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio. The temperature was kept constant at 20° ± 1°. The chromatogram was unidimensional. The solvent mixture of *n*-propyl alcohol and 0.1 *N* HCl in 2:1 ratio was stored at the above-mentioned temperature.

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[1] Cylindrical Jar, Catalog No. 805 IP. C. 12 x 24. Fisher Scientific Co., St. Louis, Missouri.

The solvent was introduced into the jar by means of a pipette, and was allowed to advance over the paper for six hours. The speed of the solvent was about 2.8 cm./hour. At the end of this period, the paper strips were dried by means of an air current, then the various reactions were conducted.

Several determinations were performed at the same time, on single compounds and on mixtures. R_F values are given in Table I.

TABLE I.—CHROMATOGRAPHY OF CHOLINE, AND THE VITAMINS B_1, B_2, NIACIN, AND NIACINAMIDE

Compound	R_F Value: Single Determination	R_F Value: Associated with Other Vitamins
Vitamin B_1	0.51	0.52
Vitamin B_2	0.39	0.39
Niacin	0.62	0.62
Niacinamide	0.44	0.44
Choline	0.59	0.60

Concentrations of the test solutions were adopted which made it possible to employ quantities of 0.01 to 0.05-ml. These quantities were introduced with 0.05-ml. pipettes graduated in 0.005 ml. Extractions of pharmaceutical preparations can conveniently be made with the above solvent. All reagents employed in this study were C. P. grade except as otherwise indicated.

Determination of Vitamin B_1.—A stock solution of thiamine hydrochloride, containing 1% of the solute, was made in *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio. Ten micrograms were placed upon the filter paper and allowed to dry. After the chromatogram was obtained the strip was dried. A solution of 2 *N* NaOH containing 5% of potassium ferricyanide, freshly prepared, was spread over the paper. A green fluorescence appeared under ultraviolet light, indicating the presence of thiamine. This reaction was found to be sensitive to 0.5 γ of thiamine.

Determination of Vitamin B_2.—The solubility of riboflavin is very low, ranging between 1 Gm.: 3,000 cc. of water and 1 Gm.: 15,000 cc. of water; variations being described to differences in internal structure (6). A stock solution of 0.25% concentration was prepared in *n* propyl alcohol and 0.1 *N* HCl in a 2:1 ratio. Warming was required. One microgram was used for the analysis. The

yellow fluorescence of the compound under ultraviolet light when it was sprayed with a solution of 0.05 *N* NaOH constituted indication of the presence of riboflavin. This reaction was found to be sensitive to 0.1 γ of riboflavin.

Determination of Niacin.—A stock solution of nicotinic acid, containing 1% of the solute, was prepared in *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio. Fifteen micrograms were used for the determination. An alcoholic solution containing 0.7% of 2,4 dinitrochlorobenzene (Eastman Kodak Company), was spread over the paper. After drying, it was heated in an oven at 100° until the strip became yellow. This was followed by spraying with an alcoholic solution containing 0.7% potassium hydroxide. After a few hours a yellow-brown spot appeared, indicating the presence of nicotinic acid.

This reaction was found to be sensitive to 10 γ of nicotinic acid.

Determination of Niacinamide.—A stock solution of nicotinic acid amide, containing 1% of the solute, was made in *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio. Niacinamide produced an intensively purple color on the paper strip when the latter was sprayed with an alcoholic solution of 2,4 dinitrochlorobenzene in 0.7% concentration, then heated at 100° in an oven. The color appeared in one hour. This reaction was found to be sensitive to 5 γ of nicotinic acid amide.

Determination of Choline.—A 1% solution of choline hydrochloride was prepared in *n*-propyl alcohol and 0.1 *N* HCl in a 2:1 ratio. Ten micrograms were used for the determination. After the paper was dried a fresh 1% aqueous solution of potassium ferrocyanide was spread over it. This solution was followed by a 0.5% aqueous solution of cobalt chloride. In a few seconds a green spot appeared, indicating the presence of choline. This reaction was found to be sensitive to 10 γ of choline.

Preparation of Radioactive Choline Acetate.—Five cubic centimeters of radioactive sodium acetate solution were placed in a 10-cc. microflask and evaporated to dryness. Three-tenths of a gram of choline hydrochloride was added, followed by 0.3 cc. of acetic acid anhydride and 0.3 cc. of acetic acid. A microcondenser was connected and the mixture was boiled for 30 minutes. The choline acetate product was washed three times with dry ether, then dried *in vacuo* at 40°. The yield was 60% of the theoretical.

Chromatography of Radioactive Choline Acetate.—Ten micrograms of a 1% radioactive choline acetate solution in anhydrous *n*-propyl alcohol were placed upon the filter paper. In order to prevent the effect of the humidity of the air, the paper was placed in a desiccator and no saturation took place in the jar. After twenty-four hours of chromatographing with anhydrous *n*-propyl alcohol, the strip was removed from the apparatus and the spot identified by its color, using the same reagent as described for choline. The area was excised and its radiation counted in a Geiger-Mueller counter.[2]

[2] Manufactured by Tracerlab Inc., Boston, Massachusetts.

A standard was prepared as follows for purposes of comparison. The same amount as used for the chromatogram was placed upon dry filter paper and measured directly. The standard solution on paper appears in Table II.

TABLE II.—CHROMATOGRAPHY OF RADIOACTIVE CHOLINE ACETATE

Type	Counts per Min.
24-hour Chromatogram 1a	35
24-hour Chromatogram 2a	40
24-hour Chromatogram 3a	37
Standard Solution on paper	744
id. after 24 hours	341
id. after 3 days	292
Background	31
Standard uranium oxide	511

In order to study the influence of the atmosphere at room temperature, the same amount was used on paper and kept in the open for twenty-four hours. The activity was also measured after three days. The standard solution on paper after twenty-four hours and after three days appears in Table II.

The great difference, between the activity of the product that was chromatographed and that of the product which was not, was due to the hydrolysis which occurred during the identification tests, preceding the counting. The reagents were spread over the paper in an aqueous medium, suitable for such a reaction. The radioactivity of a twenty-four-hour chromatogram on which no identification agent was used was found to be the same as that of the standard solution. The room temperature was 20° ± 1°; the relative atmospheric humidity 80%.

SUMMARY AND CONCLUSION

Using the paper chromatographing technique, it was found possible to set up a qualitative determination of the vitamins B_1, B_2, niacin, and niacinamide free or associated with choline. A spot test for each is described and the sensitivity of the test is stated.

The preparation of radioactive choline acetate was worked out, and the hydrolysis of the compound studied. After twenty-four hours of ordinary atmospheric interference choline acetate was hydrolyzed to the extend of 54 per cent and after three days nearly 61 per cent. It was completely hydrolyzed in an aqueous medium within a few seconds.

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A Flavor Panel Study of the Application of Monosodium Glutamate to the Flavor of Pharmaceutical Products*

By JEAN F. CAUL and EMILY L. ROCKWOOD

The value of using a small trained taste panel to define flavor problems is pointed out by a study of the effectiveness of monosodium glutamate (MSG) in improving the flavor of various fluid pharmaceutical products. The addition of monosodium glutamate appeared to reduce metallicness in iron-containing liquids, fish oil aroma in cod liver oil, and aftertaste and bitterness in a variety of preparations. Particular attention was paid to bitter taste which exemplifies a prominent taste factor often needing correction. An analytical panel method for evaluating the effect of MSG on the intensity and duration of bitter is given.

UNPLEASANT FLAVORS have always been associated with drugs and were once thought to be an indication of their efficacy. Increased interest in and information about flavor and odor in recent years have contributed impetus to the desire for making drug products more palatable and appealing. A considerable proportion of these distasteful drug preparations exhibit flavor characteristics which are resistant to the usual pharmaceutical flavoring materials. We believe that some of the difficulties encountered with such preparations can be resolved by a considered approach.

This approach is based on the concept that flavor is complex. Flavor-by-mouth is comprised of taste, aroma, and feeling. Four basic tastes are recognized: sweet, sour, salty, and bitter. These are perceived in the mouth by specialized receptors located mostly on the tongue. Aromas are perceived in the nose either by direct sniffing or, when material is taken into the mouth, in their rise from the mouth to the olfactory patch. Feeling factors are noted by both nose and mouth. A few examples of mouth feelings are astringency, chalkiness, or burning.

The complexity of flavor arises not only from the existence of vast numbers of flavor components but from the numbers in a given product that can be perceived or recognized, giving flavor identity to the product, and those which are present in subthreshold or unrecognizable quantities, contributing to the blend. The perceptibility of a flavor factor appears to depend on its relationship to the other factors or flavor notes present. Such relationships may be considered from several standpoints—such as the taste antagonism of sweet and bitter; the chemical reduction of free amine odor by acid; or the mechanism of hydrogen bonding, as yet unstudied as it pertains to flavor.

Each flavor problem is unique and requires individual treatment. Usually, it is not enough to select a flavorful vehicle for the active ingredients of a pharmaceutical preparation. Corrective agents must fit the flavor pattern of the distasteful material. A long-lasting taste, such as bitterness, should be combated with an agent that reduces duration of taste or whose intensity persists as long or longer. A strong note may be blended by the addition of a specific material that either counteracts intensity or matches it. Sometimes it is necessary to use a combination of agents. For example, in one of our hypothetical studies (1) we have found it possible to mask the intense bitterness of a water solution of quinine sulfate by adding appropriate amounts of salt, sugar, and citric acid (representing three basic tastes). The total effect of the three on bitterness was greater than that of any two, and the final mixture furnished four practically simultaneous stimuli or tastes which the tongue was unable to distinguish. This was a well-blended mixture.

A useful rule of thumb for flavor correction is as follows: to combat unpleasant taste try other tastes and demulcents; for unpleasant odors try aromatic materials and fixatives. This does not exclude recourse to official syrups, for such preparations are usually complex themselves and generally furnish at least one taste factor, several aromatics, and a feeling factor.

Official elixirs and syrups for masking both ammonium chloride and quinine were studied by Wright (2) and Purdum (3). Their methods were later combined by Boothe and Kaufman (4) to study various essential oils in appropriate vehicles for urea and quinine bisulfate. The efficacy of imitation flavors in masking ammonium chloride and quinine hydrochloride was checked by Langford and Becker (5). All of these studies required panels of relatively untrained tasters and statistical analyses of findings.

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Hamann (6) has warned of the pitfalls of misusing panels, and we agree with his criticism. Selection of corrective agents for a flavor problem is a laboratory project. Selection of a characterizing flavoring agent is primarily dependent on consumer reaction.

The objective of this article is to show how a small panel of trained tasters can be used to measure corrective action. The agent selected for this study was monosodium glutamate (MSG) whose now well-known blending and seasoning effect in foods led to the exploration of its possibilities with pharmaceutical products. Most of the distasteful factors encountered in pharmaceutical products have their counterparts in foods, many of which are favorably affected by relatively small amounts of monosodium glutamate. Parallel action might be expected especially in instances of high bitterness, metallic flavor, and long aftertaste (7, 8). Liquid preparations were studied, since, on the whole, they present more flavor problems than do solid products.

EXPERIMENTAL

Screening.—An arbitrary selection was made of current products representing what might be considered some of the outstanding types of flavor problems encountered in liquid pharmaceutical preparations. All were obtained at local drug stores. In order to dissolve monosodium glutamate, cod liver oil U. S. P. was emulsified in the laboratory according to standard technique (9).

Prior to panel testing, samples of most of the products were checked by two trained tasters in order to choose a level of monosodium glutamate which was not itself detectable by taste. Each sample containing added MSG was presented to the four-member panel with a control.

These panel members were technically trained persons, members of the Flavor Laboratory staff, who were thoroughly grounded in flavor concepts and techniques and were interested in and familiar with pharmaceutical products. They examined the aroma of the sample first and then the flavor-by-mouth, recording their findings as the differences between the sample and its control. The amounts for tasting were not measured, each taster being allowed as much as necessary for analysis. In most instances, 4 cc. was probably the maximum amount.

These flavor panel sessions were generally open ones, wherein a discussion of samples took place after individual examinations had been made. Open panels allow for the settling of any differences of opinion and for any retasting and checking that might be required.

The findings, summarized in Table I, show that monosodium glutamate has good flavor improvement potentialities for certain types of pharmaceutical products.

Metallicness typical of hematinics showed the most clear-cut response to monosodium glutamate. Reduction of the "iron-pipe" character in these products was probably due to sequestration mechanism. Earlier work not reported elsewhere showed that 0.1 and 0.2% additions of MSG to water solutions containing either 10 p.p.m. ferrous or ferric ions considerably reduced their metallic character.

It was anticipated that the saliva-stimulatory effect of MSG would be valuable in alleviating the extreme chalkiness of liquid antacids and the oily feel of mineral oil and cod liver oil emulsions. As Table I shows, any salivation effect was overridden; however, the fishiness of cod liver oil was reduced. It is quite possible that increasing the amount of MSG to be added would have resulted in perceptible improvement. However, such treatment would

TABLE I.—EFFECT OF MSG ON DISTASTEFUL QUALITIES OF A FEW LIQUID PHARMACEUTICAL PRODUCTS

Product	Predominant Flavor Problem	Added MSG, % by Wt.	Observations
Aluminum hydroxide suspension	Chalky-astringency	0.05	Not improved: salivation effect of MSG covered by chalkiness
Milk of magnesia U. S. P.	Chalky-astringency	0.05	Not improved: salivation effect of MSG covered by chalkiness
Mineral oil emulsion	Oily feel	0.05	Not improved: salivation effect of MSG covered by oiliness
Lactobacillus acidophilus	Putridness	0.05	Not improved
Ferrous gluconate elixir	Metallic	0.05	Much improved: metallic reduced and aftertaste shortened
Ferrous sulfate elixir	Metallic	0.05	Much improved: metallic reduced and aftertaste shortened
Cod liver oil emulsion	Fishiness	0.05	Improved: fish-oil aroma and flavor reduced
Vitamin mineral mixture	Yeasty and thiol	0.05	Much improved: flavor blended, metallic reduced; aftertaste cleaner and shorter
Thiamin elixir	Yeasty and thiol	0.10	Improved: slight reduction in bitterness
B Complex syrup	Long lasting bitterness	0.10	Improved: bitterness level reduced slightly; aftertaste slightly reduced in level and intensity
Liver and iron extract	Hydrolysate flavor, metallic	0.10	Improved: less metallic; aftertaste cleaner and shorter, less bitter

require blending out of the MSG taste—an experiment considered beyond the scope of this survey.

Also observed is the fact that while MSG did not appear to be effective against the yeasty-thiol flavor typical of thiamin, it reduced other flavor factors in certain of the vitamin preparations. Of particular interest was its favorable effect on bitterness in flavor-by-mouth and in aftertaste.

Study of Bitterness.—Because bitterness is a factor frequently to be contended with in flavor problems and also because it responded to MSG in the above study, it was made the subject of further study by the taste panel. Bitterness, as mentioned in the introduction, is one of the four primary tastes. Invariably, in commercial products it either is perceived along with adjunctive factors such as astringency or metallicness, or is long-lasting, persisting well into the aftertaste. Even in water solution, bitter-tasting chemicals often possess additional flavors. For example, some of the barbiturates are sharply bitter and also metallic. On the other hand, the bitterness of both quinine sulfate and sucrose octaacetate is sharp with a minimum of adjunctives. Caffeine and sucrose octaacetate exemplify long-lasting bitterness.

Such differences are very important in flavor. A quickly disappearing bitterness, even though of high intensity, is generally regarded as more tolerable than an insistent one of only moderate intensity. The Flavor Laboratory has developed a testing technique which measures intensity of a flavor factor, in this instance bitterness, with respect to its duration (10). Comparison of the resultant bitterness curves is useful in evaluating potentialities of corrective agents.

Figure 1 is a chart depicting the bitterness curves of four bitter-tasting chemicals: 0.02% quinine sulfate; 0.097% barbiturate; 1.5% caffeine; and a saturated solution of sucrose octaacetate. The *x*-ordinate represents duration marked in fifteen-second intervals, and the *y*-ordinate, intensity of bitterness designated according to the system of notation of the Favor Profile method (10). Each curve resulted from numerous panel sessions in which the members tasted the bitter solution and individually recorded findings on graph-like response sheets. The fifteen-second intervals were read from a timer in full view of the panel.

Adding 0.05 and 0.1% MSG alone to the four bitter solutions did not cause reduction in either intensity or duration of bitterness. The next step of the study tested the effect of adding MSG to bitter substances presented in 60% sucrose solution. Typical findings with the barbiturate are given in Fig. 2.

Two conclusions may be drawn. First, the addition of MSG to the bitter barbiturate in a simple syrup caused desirable reduction of bitter intensity and persistence. Secondly, the sucrose solution alone perceptibly reduced the bitter taste at first, probably by virtue of its sweetness and demulcent-like action, and then appeared to prolong duration of the bitterness, probably because of high viscosity.

This study throws additional light on the role of MSG. Its bitter-reducing effect was demonstrable not when used alone but when used in conjunction with a sweet, viscous solution. Common knowledge has it that sweetness and bitterness are antagonists, and also that MSG may enhance sweetness. Our earlier work has shown that such sweetness enhancement occurs at low levels (0.6–1.8%) of sucrose, and it is doubtful that with a 60% sucrose solution an important increase of sweet taste would result from MSG. Rather it is inferred that the blending action of MSG bears the responsibility for bitterness reduction depicted in Fig. 2.

SUMMARY

An introductory study of the "glutamate" effect on pharmaceutical-type flavors has been presented in order to demonstrate the value of deliberate approach and use of a small trained taste panel in correction of distasteful flavors. The first portion of the study surveyed the potentialities of monosodium glutamate in combating a few typical flavor problems of currently marketed

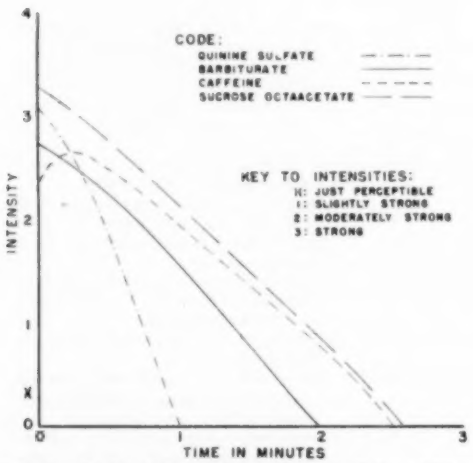

Fig. 1.—Rate of taste disappearance of four bitter substances in solution.

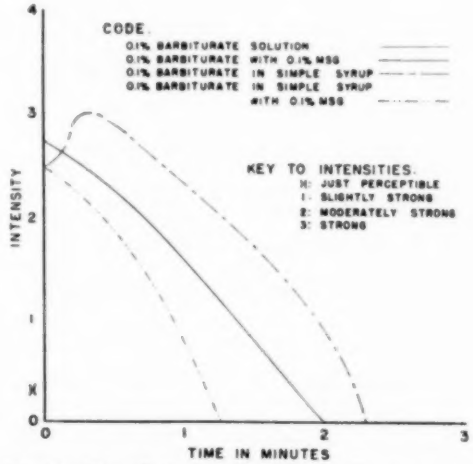

Fig. 2.—The effect of 0.1% MSG on disappearance of bitter taste in water and in simple syrup.

fluid products. It was found that MSG holds promise for reducing metallicness in iron-containing liquids, fish oil aroma in cod liver oil, and aftertaste and bitterness in a variety of preparations.

The second part suggested a means of evaluating a single factor, in this case bitterness and its taste reaction to MSG. Three types of bitter tastes were introduced: short-lived (quinine sulfate); long-lived (caffeine and sucrose octaacetate); and relatively long-lived, having accompanying metallic flavor (barbiturate). No reduction of bitterness was found when small quantities of MSG were added to water solutions of these four chemicals. But in sucrose solution, the barbiturate bitter responded favorably to MSG. This was demonstrated by panel-drawn graphs showing reduction in intensity and duration of bitterness. The action of MSG in this instance was thought to be that of a blender.

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Local Anesthetic Activity and Toxicity of Several Esters of *p*-Tertiary-Butylbenzoic Acid*

By LAMAR B. DALE, Jr.,† and ELBERT VOSS‡

Studies of the local anesthetic activity, irritancy, and toxicity were made of four esters of *p*-tertiary-butyl-benzoic acid, namely the β- and γ-diethylaminopropyl, the β-diethylaminoethyl, and the piperidinoethyl *p*-tertiary-butyl-benzoates. The compounds were found to be more active as surface anesthetics on mucous membranes than cocaine, more active in infiltration anesthesia than procaine, and of approximately the same order of activity as conduction or nerve block anesthetics as procaine. The esters were found to be far more irritant to mucous membranes than cocaine and more irritant than procaine upon parenteral injection. The acute intraperitoneal toxicities of the experimental compounds were found to be comparable to that of procaine. The γ-diethylaminopropyl ester appeared to be the most promising for further studies in local anesthesia.

BRYAN (1) has synthesized various esters of *p*-tertiary-butylbenzoic acid with the object of producing compounds with local anesthetic activity. This synthesis paralleled the earlier work of Bryan and Foote (2) on *p*-isopropylbenzoic acid.

The present investigation was carried out to extend the knowledge of the pharmacology of these compounds, limiting the research to four derivatives, namely the β- and γ-diethylaminopropyl, and the β-diethylaminoethyl, and the piperidinoethyl-*p*-tertiary-butyl-benzoates. Experiments were conducted to investigate the local anesthetic action, irritancy, and toxicity of the compounds.

EXPERIMENTAL

Preparation of Materials.—All solutions used were made fresh daily by dissolving the hydrochloride in distilled water or normal saline. The *p*H of the resulting solutions fell within the range of 5.5 to 6.5 in all cases.

Surface Anesthesia.—Guinea pigs weighing 700 to 900 Gm. were employed. The animals were restrained by placing them in small, individual boxes equipped with a lid and a V-shaped opening at one end to allow only the head to remain free.

The solutions of local anesthetics were applied to each eye of the guinea pigs with a medicine dropper by a modification of the method of Chance and Lobstein (3).

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Three concentrations, spaced equidistant on the logarithmic scale, were used for each compound with a minimum of 10 animals per concentration. Each eye was considered individually, thus giving 20 eyes per concentration.

The stimulus was applied to the center of the cornea with an L-shaped glass rod, of small diameter with rounded end. The eye was approached from the side and rear with care not to cause a blink by coming into contact with the eyelashes.

The first stimulation was applied to the cornea five minutes from the time of instillation of the anesthetic solution. The stimulation was repeated at five-minute intervals on each eye. The duration of anesthesia was recorded as the last positive reading.

In order to compare the potencies of the compounds with each other and with procaine hydrochloride and cocaine hydrochloride the method of Luduena and Hoppe (4) of threshold anesthetic concentrations (TAC) was employed. The comparison appears in Table I.

TABLE I.—CORNEAL ANESTHESIA

Compound	TAC_5, %	Slope,[a] Min.	Cocaine Ratio
β-Diethylaminoethyl	0.274	7.05	2.4
β-Diethylaminopropyl	0.128	5.41	5.1
γ-Diethylaminopropyl	0.090	8.28	7.2
Piperidinoethyl	0.520	9.76	1.3
Procaine	2.200	7.75	0.3
Cocaine	0.650	9.68	1.0

[a] Average increase in duration obtained by doubling the concentration.

Infiltration Anesthesia.—Adult male guinea pigs weighing 700 to 900 Gm. were used in this test. Areas 6 to 7 cm. in diameter were clipped in the center of the back. It was found that if fine-bladed clippers were used the areas need not be shaved and no interfering irritation was produced. This permitted the preparation of the animals on the day of the test.

According to Bulbring and Wajda (5) the sensitiveness of the skin is greatest in the middle and slightly more so in the front area than in the back area. They compensated for this by testing each concentration of the local anesthetic in both front and back regions. From preliminary experiments in the present study it was found that more uniform results could be obtained by making the injections just to the right or left of the midline about 5 cm. caudad from the last cervical vertebra. All injections were made intracutaneously using a number 26 gauge, $^3/_8$-inch hypodermic needle. The dose was contained in 0.25 cc. normal saline. The wheal was outlined in ink immediately after being raised.

Three graded concentrations were used for each compound with a minimum of 10 animals per concentration. The guinea pigs were firmly held during the injections and in the subsequent stimulation of the wheals. The holding of the animal in the hand during stimulation was found to be superior to other methods of restraining for tremors in response to the shock could often be felt when they could not otherwise be detected.

The stimulus giving the most consistent response was found to be a 20-volt Faradic shock. Pin-prick stimulation or electric stimulation of just threshold value would not give rise to a uniform reaction even in unanesthetized areas.

Readings were taken every five minutes, starting five minutes after injection. The duration of anesthesia was given as the last positive reading. Regression lines were calculated as for surface anesthesia and the threshold was selected as the concentration that would be expected to produce anesthesia for the average duration of five minutes (threshold anesthetic concentration five minutes TAC_5). The results are contained in Table II.

TABLE II.—INFILTRATION ANESTHESIA

Compound	TAC_5, %	Slope,[a] Min.	Procaine Ratio
β-Diethylaminoethyl	0.082	17.6	2.7
β-Diethylaminopropyl	0.077	18.4	2.9
γ-Diethylaminopropyl	0.072	16.6	3.1
Piperidinoethyl	0.002	3.8	112.0
Procaine	0.224	12.5	1.0

[a] Average increase in duration obtained by doubling the concentration.

Conduction Anesthesia.—The procedure used was essentially that of Shackell (6). The duration of anesthesia was based on motor paralysis by the method of Loomis and Spielmeyer (7).

In order to compare the potencies of the compounds with that of procaine, the concentration of each was varied until the duration of anesthesia produced was equal to that produced by a 0.5% solution of procaine. In each case, with the exception of procaine, 5 animals were used for each concentration tested. Ten animals were used for the determination of procaine. The resulting data appear in Table III.

TABLE III.—CONDUCTION ANESTHESIA

Compound	Concentration, %	Duration, Min.	Procaine Ratio
β-Diethylaminoethyl	0.5	31 ± 4.58	1.00
β-Diethylaminopropyl	1.0	32 ± 5.10	0.50
γ-Diethylaminopropyl	0.5	30 ± 5.00	1.00
Piperidinoethyl	1.5	31 ± 5.77	0.33
Procaine	0.5	28 ± 4.30	1.00

Parenteral Irritation.—In order to test the irritation produced by the compounds after parenteral administration the trypan blue test as devised by Hoppe, Alexander, and Miller (8) was employed. Adult albino rabbits, sexed at random, weighing from 2 to 3 Kg. were used.

In order to obtain linear regression lines and thus allow graphic analysis of the irritation data, the scoring plan of Luduena and Hoppe (4) was used.

Each compound was injected at three or more concentrations, spaced equidistant on the logarithmic scale, into ten test areas per concentration distributed among ten different animals. The irritation scores were averaged for the respective con-

centrations at each reading to obtain the maximum average irritation score for each concentration occurring during the test period. The data are contained in Table IV.

Mucous Membrane Irritation.—A test for irritation to mucous membranes was carried out by the rabbit eye irritation method of Hoppe, Alexander, and Miller (8).

TABLE IV.—PARENTERAL IRRITATION

Compound	TIC,[a] %	Procaine Ratio
β Diethylaminoethyl	0.130	47
β Diethylaminopropyl	0.070	87
γ Diethylaminopropyl	0.115	53
Piperidinoethyl	0.078	79
Procaine	6.100	1

[a] Threshold irritant concentration.

The local anesthetics were dissolved in distilled water immediately before instillation into the conjunctival sac of one eye, leaving the other as control. The treated eye was examined in comparison with the control eye at one, two, four, and eight hours after medication and graded according to the method of Draize, *et al.* (9).

Tests were made of 2 or more concentrations, each succeeding concentration being twice that of the immediately preceding concentration, of each local anesthetic, using 5 rabbits per concentration.

The values obtained from the 5 animals were averaged at each reading and the maximum values were used to obtain the threshold irritation score. The threshold irritation concentration (TIC) for each compound was taken as that concentration, expressed in per cent, which produces no more than mild irritation. The data appear in Table V.

TABLE V.—MUCOUS MEMBRANE IRRITATION

Compound	TIC,[a] %	Cocaine Ratio
β-Diethylaminoethyl	1.0	16
β Diethylaminopropyl	1.0	16
γ-Diethylaminopropyl	1.0	16
Piperidinoethyl	0.5	32
Cocaine	16.0	1

[a] Threshold irritant concentration.

Acute Toxicity.—The acute intraperitoneal toxicities of the experimental compounds and of procaine were determined in albino mice.

Male, Swiss strain, albino mice weighing 18–20 Gm. were used in the test. Ten animals were used per dosage level with a minimum of three levels per compound. The volume of the injection was kept between 0.2 and 0.4 cc.

The animals were kept in an air-conditioned room during the test with the temperature maintained at 22°C. Observations were made at three, twelve, thirty-six, and seventy-two hours. The per cent mortality at seventy-two hours was converted into probits and plotted against the logarithm of the dose. The LD_{50} of each compound was then calculated by the method of Miller and Tainter (10).

TABLE VI.—ACUTE TOXICITY

Compound	LD_{50}, Mg./Kg.	Procaine Ratio
β Diethylaminoethyl	214 ± 22	0.86
β Diethylaminopropyl	159 ± 20	1.16
γ Diethylaminopropyl	245 ± 8	0.75
Piperidinoethyl	145 ± 26	1.28
Procaine	185 ± 7	1.00

SUMMARY

1. Studies of the local anesthetic activity, irritancy, and toxicity were made of four esters of *p*-tertiary-butylbenzoic acid, namely the β- and γ-diethylaminopropyl, the β-diethylaminoethyl, and the piperidinoethyl *p*-tertiary-butylbenzoates.

2. The compounds were found to be more active as surface anesthetics on mucous membranes than cocaine, more active in infiltration anesthesia than procaine, and of approximately the same order of activity as conduction or nerve block anesthetics as procaine.

3. The esters were found to be far more irritant to mucous membranes than cocaine and more irritant than procaine upon parenteral injection.

4. The acute intraperitoneal toxicities of the experimental compounds were found to be comparable to that of procaine.

5. The γ-diethylaminopropyl ester appeared to be the most promising for further studies in local anesthesia.

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Nonaqueous Titration of Antibiotic Substances*

By CATHERINE N. SIDERI† and ARTHUR OSOL

Antibiotic substances, whether present as the base or as the hydrochloride salt, as well as their dosage forms, may be assayed in a medium containing glacial acetic acid by titration with a volumetric solution of perchloric acid in dioxane.

THE UTILITY of nonaqueous titration methods for the analysis of medicinal substances is apparent from the large number of reports which have appeared, especially in the recent literature, on this subject. An excellent review of the principles of such methods, as well as of the pertinent literature, has been prepared by Pifer, Wollish, and Schmall (1). Reference is made in their paper to the possibility of assaying certain antibiotic bases and their salts, and to the actual titration of Aureomycin hydrochloride, first performed in our laboratory and described in detail in this paper. Ekeblad (2) reported obtaining good results in the titration of Terramycin hydrochloride, using a solution of perchloric acid in acetic acid as the titrant, but he was unable to obtain reproducible results with Aureomycin hydrochloride because of incomplete solution of the latter in his analytical procedure.

We report here the results of our studies of the titration of Aureomycin hydrochloride, Ilotycin, Magnamycin base and hydrochloride, and Terramycin hydrochloride. Results on certain dosage formulations of Aureomycin hydrochloride are also given.

EXPERIMENTAL

Reagents.—Perchloric acid volumetric solutions of 0.05 *N* and 0.1 *N* concentration, in dioxane, were prepared and standardized as described in the paper of Kleckner and Osol (3), the dioxane first having been purified to remove color-producing impurities by shaking with asbestos (4).

Procedure.—The bulk antibiotic samples were usually dried, either by heating at 110° to constant weight, or in an Abderhalden drier over sulfuric acid, at the temperature of water boiling under atmospheric pressure, with the drying chamber evacuated to a pressure of approximately 75 mm. of mercury. In some cases the water content of the samples was determined separately and the analytical data corrected to a dry weight basis of the sample.

From 300 to 1,000 mg. of bulk antibiotic (300 mg. of Aureomycin hydrochloride, because of its low solubility), accurately weighed, was dissolved in 80 cc. of glacial acetic acid; heating on a boiling water bath to effect solution was essential in the case of Aureomycin hydrochloride. Where halide salts were involved, 10 cc. of 6% (w/v) solution of mercuric acetate in glacial acetic acid was added, and the solution titrated with 0.05 *N* or 0.1 *N* perchloric acid, depending on the weight of sample employed. The end point of the titration was determined potentiometrically, using conventional glass and calomel electrodes, although in most titrations 0.5 cc. of a 0.1% solution of crystal violet in glacial acetic acid was added in order to observe the color changes accompanying the potential change.

Potential changes at the equivalence point were found to be of the order of 30 to 60 millivolts per 0.1 cc. of 0.1 *N* titrant. These were of such a magnitude as to permit direct observation of the end point, without having to plot the voltage data; a better estimate of the equivalence point was possible when the point of maximum change of potential was observed from a graphical plot of the data. Reproducibility of titration data, under the latter conditions, was of the order of 0.25%, even though the volume of titrant employed was generally somewhat less than 10 cc. (a 10-cc. burette, graduated at intervals of 0.05 cc. was employed). The fact that the volume of a drop of titrant is about half that of an aqueous solution released from the same orifice is an obvious advantage in obtaining good precision.

While crystal violet was added as an indicator to most of the solutions titrated, it was apparent that the color change was not sufficiently sharp to permit of as high a degree of precision in determining the equivalence point as when the point of inflection of the potential curve was determined.

All of the antibiotic substances titrated reacted as monoacidic bases. The molecular weight of three of the antibiotics is known; that of Magnamycin had not been established with certainty at the time of writing this report. In the case of Magnamycin the equivalent weight of a highly purified sample of the base was found to be 843 ± 4; this is in good agreement with the formula $C_{42}H_{69}NO_{18}$ (molecular weight 843.98), which is under consideration as a possible empirical formula for Magnamycin (5). The results of our titrations of the other antibiotics are given in Table I.

Assay of Dosage Forms.—Dosage forms of the antibiotics investigated in this study may also be assayed by the procedure given above, or by a modification of it. Since it is known that stearates, commonly employed as lubricants in tablets, will react with perchloric acid, the analytical procedure when a stearate is present needs to be modified, as suggested by Pifer and Wollish (6), by first titrating the sample in the absence of mercuric acetate, then determining the additional volume of perchloric acid required after the mercury salt has

* Received July 8, 1953, from the Chemistry Department of the Philadelphia College of Pharmacy and Science, Philadelphia.

This paper reports certain observations made in the course of research on nonaqueous titration methods performed by Catherine N. Sideri in partial fulfillment of requirements for the degree of Doctor of Science, at the Philadelphia College of Pharmacy and Science.

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been added; the additional volume of perchloric acid required is a measure of the hydrogen chloride associated with the antibiotic base.

The fact that the hydrogen chloride component is the one actually determined in this instance is, of course, a disadvantage of such a differential method of titration as compared with the otherwise similar procedure employed in the absence of stearates. In that case the volume of perchloric acid solution required is determined solely by the amount of antibiotic base present and is independent of the amount of hydrogen chloride associated with it.

TABLE I.—NONAQUEOUS TITRATION OF ANTIBIOTIC SUBSTANCES[a]

Antibiotic	Formula	Assay Average, %
Aureomycin hydrochloride (lot No. 1)	$C_{22}H_{23}N_2O_8Cl \cdot HCl$	99.7
Aureomycin hydrochloride (lot No. 2)	$C_{22}H_{23}N_2O_8Cl \cdot HCl$	99.4
Aureomycin hydrochloride (lot No. 3)	$C_{22}H_{23}N_2O_8Cl \cdot HCl$	99.6
Ilotycin	$C_{29}H_{72}NO_{13}$	100.2
Terramycin hydrochloride	$C_{22}H_{24}N_2O_9 \cdot HCl$	99.2

[a] We are indebted to Lederle Laboratories Division, American Cyanamid Company, for supplies of Aureomycin hydrochloride, to Eli Lilly and Company for Ilotycin, and to Chas. Pfizer and Company for both Terramycin hydrochloride, and Magnamycin base and hydrochloride.

In the case of ointments containing Aureomycin hydrochloride we found that a mixture of 40 cc. of glacial acetic acid and 60 cc. of chloroform served to dissolve both the antibiotic and the ointment base in as much as 7 Gm. of ointment (containing 3% of Aureomycin hydrochloride) when heated on a water bath. The titrant employed was either 0.05 *N* or 0.1 *N* perchloric acid. The small volume required (generally less than 5 cc.) demanded the most careful technique to be employed in order to obtain results of fairly good precision (approximately 1%). Thus a 5-cc. burette, which can be read to 0.01 cc. without difficulty, is essential for titrations of this kind.

In the assay of suppositories containing Aureomycin hydrochloride it was found that digestion at room temperature, for about thirty minutes, with 20 cc. of chloroform, followed by addition of 60 cc. of glacial acetic acid and heating of the mixture on a water bath until it becomes clear, permitted satisfactory titration of the antibiotic. Mercuric acetate must be added to this solution, and also to that of the ointment, prior to titration with perchloric acid.

The results of the assay of these dosage forms of Aureomycin hydrochloride are given in Table II.

TABLE II.—TITRATION OF DOSAGE FORMS OF AUREOMYCIN HYDROCHLORIDE

Dosage Form	Content of Antibiotic	Assay Result
Ointment (lot No. 1)	3%	2.96%
Ointment (lot No. 2)	3%	2.92%
Suppository (No. 1)	100 mg. per suppository	103.6 mg.
Suppository (No. 2)	100 mg. per suppository	102.8 mg.

SUMMARY

1. The antibiotic substances Aureomycin, Ilotycin, Magnamycin, and Terramycin, whether in the form of the base or hydrochloride salt, may be quantitatively titrated, following solution in glacial acetic acid, with a volumetric solution of perchloric acid in dioxane.

2. Dosage forms, including ointments and suppositories of the same antibiotics, may be assayed for content of antibiotic by the same method or a slight modification thereof.

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Compressed Tablets—Average Weight*

By ANGEL S. ARAMBULO† and DWIGHT L. DEARDORFF‡

Sodium chloride granules were separated into narrow-size ranges and compressed into tablets on a single-punch tablet press. The press adjustments were maintained constant throughout a given compression series. Granules ranging in size from 4,380 to 81 microns were compressed into tablets with varying degrees of success. As the particle size decreased, the average tablet weight of the batches of tablets increased, passed through a maximum at 150 to 350 microns, and then decreased. A similar trend in the physical quality of the tablets was noted.

Powders to be compressed into tablets usually require a treatment which transforms the mixed powders into free-flowing granules. Granulations, according to those individuals skilled in the art, should consist of particles rather uniform in size in order to produce tablets with uniformity in properties, such as appearance, average weight, weight variation, hardness, rate of disintegration, and dosage (1). It is generally believed that "too large" or "too small" granules will cause difficulty during compression.

The literature does not, however, define clearly the relationship between the distribution of particle size in tablet granulations and the physical properties of the tablet. The purpose of this investigation was to study the effect of the particle size on the average weight of sodium chloride tablets.

EXPERIMENTAL

Granules of U. S. P. sodium chloride were prepared by slugging, comminution, and sieving, by means of a modification of the ASTM method for sieving refractory materials, to obtain relatively homogeneous fractions approximately 99% of the size listed in Table I (2). The average of the sieve openings of the U. S. standard sieves through which the material passed and on which it was retained, was taken as the average particle size. No lubricants or disintegrants were used.

Table I.—Particle Size, Average Weight, and Physical Appearance of Tablets from Manual Operation

Sieve No.		Average Particle Size, Microns	Average Weight, Gm.	Physical Appearance
Passed Through	Retained On			
4	5	4,380	0.83	Crumbling, chipped, mottled
5	6	3,680	0.85	Crumbling, chipped, mottled
6	7	3,095	0.82	Crumbling, chipped, mottled
7	8	2,605	0.87	Crumbling, chipped, mottled
8	10	2,190	0.89	Crumbling, chipped, mottled
10	12	1,840	0.86	Crumbling, chipped, mottled
12	14	1,545	0.87	Crumbling, chipped, mottled
14	16	1,300	0.87	Chipped, mottled
16	18	1,095	0.89	Chipped, mottled
18	20	920	0.89	Chipped, mottled
20	25	775	0.91	Mottled
25	30	650	0.96	Glossy, well-formed
30	35	545	0.98	Glossy, well-formed
35	40	460	1.02	Glossy, well-formed
40	45	385	1.00	Glossy, well-formed
45	50	324	1.02	Glossy, well-formed
50	60	274	1.00	Very glossy, well-formed
60	70	230	0.99	Very glossy, well-formed
70	80	194	0.97	Glossy, well-formed
80	100	163	0.95	Glossy, well-formed
100	120	137	0.91	Glossy, well-formed
120	140	115	0.87	Glossy, well-formed
140	170	97	0.83	Glossy, well-formed
170	200	81	0.78	Glossy, chipped

Manual Compression of Small Samples.—Small samples of the 24 fractions isolated were compressed in a single-punch tablet press using $^7/_{16}$-inch punches. The press was operated manually and the press adjustments were maintained constant throughout the experiment. The material was fed into the die with a spatula and care was taken to prevent packing of the material in the die.

* Received August 22, 1952, from the Department of Pharmacy, College of Pharmacy, University of Illinois, Chicago.

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This paper is based on a thesis submitted by Angel S. Arambulo to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Master of Science.

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Table I and Fig. 1 give the average weights of the tablets so compressed and data on their physical appearance.

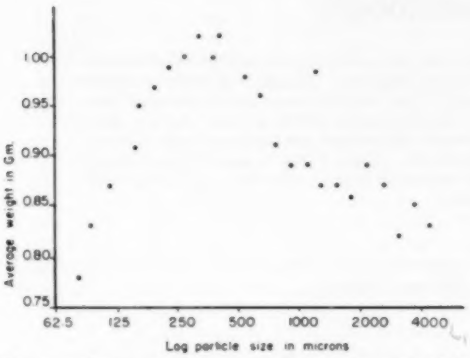

Fig. 1.—Average weight *vs.* particle size of tablets from manual operation.

Power Compression.—This phenomenon was investigated further, using 500-Gm. batches of certain granulation sizes. The same press and punches were used and the new press adjustments were held constant throughout the series. The press was operated at a speed of about 100 tablets per minute. The tablets were collected into 24–28 samples in the order of production. From each sample, four apparently perfect tablets were selected and weighed.

Errors such as those resulting from shifts in machine adjustment or substantial internal flow of granulation (3) were minimized by the preparation of statistical quality control charts for all batches.

Table II and Fig. 2 give the average weights of the batches.

The tablets made by power compression exhibited maximum weight at 150–350 μ. As the weight increased, the percentage of physically perfect tablets increased; the tablets became more glossy and in general more satisfactory. This trend also passed through a maximum followed by production of tablets of lesser quality.

Granules as large as 3,095 μ were compressed into tablets by suitable adjustment of the press. As the granule size increased above about 1,000 μ, however, mottled tablets were obtained. As the granule size increased above approximately 2,000 μ, the difficulty of obtaining physically perfect tablets increased until with 3,000 μ granules, 20–30% of the tablets had rough edges or were in some way imperfect.

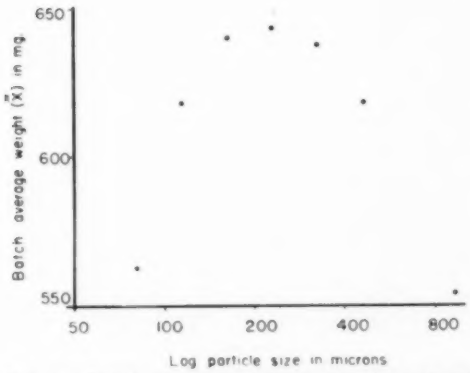

Fig. 2.—Batch average weight *vs.* particle size of tablets from power operation.

TABLE II.—PARTICLE SIZE AND AVERAGE WEIGHT OF TABLETS FROM POWER OPERATION

Average Particle Size, Microns	Average Weight, Mg.
920	554.0
460	617.7
324	638.0
230	642.8
163	641.4
115	618.0
81	562.9

DISCUSSION

Large granules give large proportions of void space in the die before compression and relatively lightweight tablets. As the granule size is reduced, the average tablet weight increases, presumably due to the decrease in void space. For still smaller particle sizes the average weight tends to decrease.

This latter trend is apparently connected with the phenomenon of bulk density. It is probably affected by changes in several factors, including occluded air, the size and shape of the particles, the coefficient of friction between particles, and agglomeration (4–6).

A distinction should be made between the variation of weight between batches studied here and weight variation within batches as described by Arambulo, *et al.* (7).

SUMMARY

1. The average weight was determined for $^{7}/_{16}$-inch tablets made on a single-punch tablet press from sodium chloride granulations of relatively uniform particle size.

2. As the particle size decreased, the average tablet weight increased, passed through a maximum at 150–350 microns and decreased.

3. As the particle size decreased, the percentage of physically perfect tablets increased, the tablets became more glossy and in general more satisfactory. This trend also passed through a maximum followed by production of tablets of decreasing quality.

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Compressed Tablets—Weight Variation*

By ANGEL S. ARAMBULO,† HELENA SUEN FU‡ and DWIGHT L. DEARDORFF§

The effect of granulation particle size on the weight variation of compressed tablets was studied, using statistical quality control procedures. Batches of lactose-starch granulation particles ranging in size from 3,045 to 324 microns and separated into narrow-size ranges were compressed in a single-punch tablet press. As the particle size decreased, the tablet weight variation decreased, passed through a minimum at 400 to 800 microns and then increased. Data from a somewhat similar experiment involving sodium chloride was found to give a minimum at 200 to 500 microns.

FOR MANY YEARS the literature of manufacturing pharmacy has advocated the control of the size of tablet granulations so as to effect an even flow of material into the machine, the production of well-formed tablets, and to safeguard the products from weight variation (1). To insure these properties, the literature provides schedules of sieves for given sizes of dies (2). These schedules define the upper limits of particle sizes and suggest lower limits by indefinite restrictions on the proportions of inadequately described "fines." They do not, however, define clearly the optimum size distribution of the mixture or the penalties for deviating from the general recommendations.

The purpose of this work was to investigate the influence of the size of granulation particles on the variation in weight of compressed tablets.

EXPERIMENTAL

Lactose-Starch Granulation.—A lactose-starch (10:1) placebo formulation granulated by the wet method using 10% starch paste was employed. The dry granulation was fractionated into the size ranges shown in Table I, after which lubricants (talc 2.6%, magnesium stearate 0.6%) and a disintegrant (starch 4.3%) were added. The average of the sieve openings of the U. S. standard sieves through which the material passed and on which it was retained was taken as the average particle size.

The compression of tablets weighing 500–700 mg. was carried out in a power-driven single-punch tablet press using $^{7}/_{16}$-inch punches from batches of 250–500 Gm. The tablets were collected into 20–24 samples (n) in the order of production. From each sample, four apparently perfect tablets were selected and weighed.

Table I and Fig. 1 permit a comparison of weight variation by means of the standard deviation of the subgroup mean weight ($\bar{X}$), corrected to that for 500 mg. tablets.

$$s = \sqrt{\frac{\sum \bar{X}^2 - \frac{(\sum \bar{X})^2}{n}}{n-1}}$$

TABLE I.—LACTOSE-STARCH TABLETS, WEIGHT VARIATION AND PARTICLE SIZE

Average Particle Size, Microns	Average Weight, Mg.	Standard Deviation, Subgroup Mean Weight (Cor.), Mg.
2,605	501.7	24.9
2,190	548.2	17.8
1,840	544.3	13.0
1,300	535.1	14.8
1,095	533.5	8.1
855	541.6	6.1
545	651.0	2.6
324	676.9	20.1

As the particle size decreased, the tablet weight variation decreased, passed through a minimum and then increased. The minimum weight variation is apparently obtained from particles 400–800 μ in size for this material and these compression conditions.

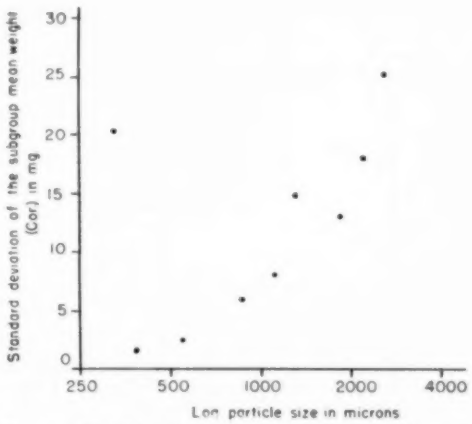

Fig. 1.—Weight variation *vs.* particle size of lactose starch tablets.

* Received August 22, 1952, from the Department of Pharmacy, College of Pharmacy, University of Illinois, Chicago.

This paper is based on portions of the theses submitted by Angel S. Arambulo and Helena Suen Fu to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degrees of Master of Science.

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TABLE II.—SODIUM CHLORIDE TABLETS, SUBGROUP AVERAGE WEIGHT AND RANGE

Subgroup No.	81 micron $\bar{X}$, Mg.	81 micron R, Mg.	115 micron $\bar{X}$, Mg.	115 micron R, Mg.	163 micron $\bar{X}$, Mg.	163 micron R, Mg.	324 micron $\bar{X}$, Mg.	324 micron R, Mg.	230 micron $\bar{X}$, Mg.	230 micron R, Mg.	460 micron $\bar{X}$, Mg.	460 micron R, Mg.	920 micron $\bar{X}$, Mg.	920 micron R, Mg.
1	584.7	34.0	638.5	61.6	652.5	69.8	645.1	20.6	651.6	37.4	623.7	7.2	561.7	70.0
2	586.7	25.6	636.5	88.4	631.7	13.6	636.5	5.0	646.4	16.8	622.8	8.2	543.7	50.8
3	559.7	51.4	636.0	69.4	642.1	30.6	638.5	5.0	645.3	15.2	623.3	3.4	530.9	33.2
4	553.6	35.0	629.9	62.0	639.7	6.0	637.8	2.8	649.0	24.4	622.3	5.4	544.8	73.6
5	554.2	20.2	615.4	60.8	649.7	34.2	638.8	5.4	645.4	8.2	620.4	9.2	559.6	46.6
6	551.1	23.8	615.3	33.6	646.7	22.8	638.2	4.4	641.5	5.6	622.7	8.8	552.3	58.8
7	550.8	39.4	611.5	29.6	638.7	13.6	638.9	6.0	647.3	13.8	621.0	10.8	549.6	71.0
8	555.7	22.4	624.5	67.6	634.5	12.4	640.5	8.0	643.3	8.2	618.6	15.8	572.5	32.8
9	543.9	39.6	611.6	36.4	636.9	12.0	639.0	3.6	641.9	2.8	619.9	6.0	533.9	60.2
10	563.0	15.4	601.9	5.4	633.7	4.2	639.9	5.0	643.6	6.4	619.5	10.0	576.3	17.2
11	573.9	18.8	619.9	75.6	646.6	24.8	637.7	8.0	643.8	8.6	621.5	4.2	551.4	36.8
12	549.4	32.2	604.7	24.4	655.5	70.6	638.9	1.8	644.9	6.6	615.8	7.8	560.1	50.0
13	553.4	29.6	603.4	4.0	637.7	9.0	639.7	4.0	642.3	2.4	621.5	11.4	555.4	33.4
14	565.9	42.4	615.3	40.6	637.2	5.0	636.8	5.8	640.9	8.0	619.7	5.6	560.1	65.8
15	564.5	62.4	599.8	12.2	639.8	3.6	639.6	5.8	640.5	6.6	619.1	7.6	572.0	36.6
16	567.4	25.4	608.8	31.4	633.0	12.6	638.9	4.0	640.2	9.6	618.4	7.6	551.6	63.0
17	559.7	53.2	633.2	71.8	654.0	30.0	637.2	5.4	639.7	7.4	614.5	7.6	545.8	55.4
18	568.4	23.0	613.7	64.0	639.2	27.0	636.8	7.0	643.8	7.2	614.6	10.8	575.3	40.0
19	571.5	60.8	629.1	55.0	636.6	2.6	635.3	11.2	639.4	8.2	612.8	16.8	535.4	14.2
20	563.2	23.4	625.3	57.2	644.7	15.4	636.5	6.6	644.6	7.2	612.7	5.4	544.9	59.8
21	572.6	13.2	623.1	64.0	648.9	24.0	635.7	2.0	642.7	7.4	611.4	5.8	564.2	65.6
22	557.1	24.6	606.6	17.8	642.6	42.0	637.2	5.0	640.1	3.4	613.1	5.2	548.0	52.4
23	559.1	53.0	634.6	72.6	639.3	20.0	634.8	5.6	639.1	2.6	613.4	5.2	539.9	77.6
24	569.2	28.2	594.5	22.8	642.2	28.0	633.8	4.0	629.5	25.4	617.6	9.8	545.7	23.8
25	574.9	57.6	...	...	632.0	44.2	...	...	...	...	603.1	32.0	574.1	50.2
26	...	...	...	...	...	...	...	...	...	...	...	...	559.3	42.0
27	...	...	...	...	...	...	...	...	...	...	...	...	559.1	67.6
28	...	...	...	...	...	...	...	...	...	...	...	...	545.0	65.8
Av.	652.9	34.2	618.0	48.5	641.4	23.1	638.0	5.9	642.8	10.4	617.7	9.1	554.0	50.5

Sodium Chloride Granulation.—The data from the power compression of sodium chloride granules, Arambulo and Deardorff (3), were analyzed for weight variation within batches, as shown in Table II and Fig. 2.

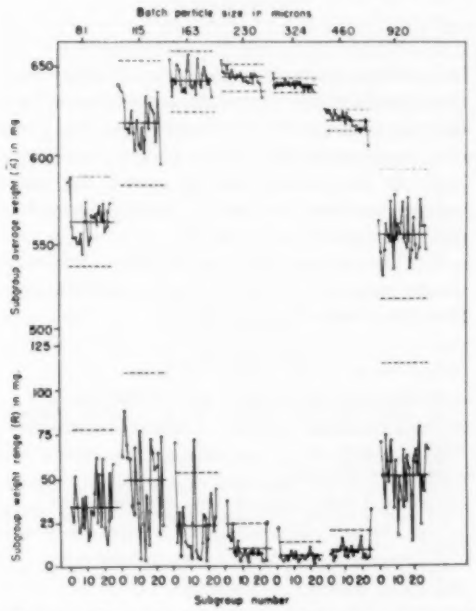

Fig. 2.—Control chart for weight averages and ranges of sodium chloride tablets.

Table II gives the values for the subgroup average weight and range and grand averages for the several particle sizes.

Figure 2 gives statistical quality control charts for the sodium chloride tablets based on the data of Table II (4). These control charts offer a visual approach to the determination of the relationship of particle size to weight variation.[1]

In the control charts each point represents the average weight or the weight range of the 4 tablets in a subgroup. These values are plotted in sequence. The lines connecting the points serve only to aid in visualization of the trend. The solid lines give the grand average weight ($\bar{\bar{X}}$) or the average range ($\bar{R}$) of the batch. The control limits are shown by intermittent horizontal lines. The upper and lower control limits for average weight were calculated using the formulas: $UCL_{\bar{X}} = \bar{\bar{X}} + A_2\bar{R}$ and $LCL_{\bar{X}} = \bar{\bar{X}} - A_2\bar{R}$. A_2 is a constant which for subgroups of 4 has the value of 0.73 (4). The upper control limits for range were computed from the formula: $UCL_R = D_4\bar{R}$. The constant D_4 has the value 2.11 for subgroups of 4.

Table III and Fig. 3 give a comparison of the weight variation for the different batches based on the standard deviation of the subgroup mean weight. Again, as the particle size decreased, the tablet weight variation decreased, passed through a minimum, and increased. For this material and these compression conditions, the minimum weight variation is apparently obtained from granules 200–500 μ in size.

[1] The authors gratefully acknowledge suggestions on control charts made by Miss Rose Grundman of the University of Illinois, College of Pharmacy.

TABLE III.—SODIUM CHLORIDE TABLETS, WEIGHT VARIATION AND PARTICLE SIZE

Average Particle Size, Microns	Average Weight, Mg.	Standard Deviation, Subgroup Mean Weight (Cor.), Mg.
920	554.0	11.51
460	617.7	3.93
324	638.0	1.78
230	642.8	3.28
163	641.4	5.36
115	618.0	10.48
81	562.9	9.56

DISCUSSION

Large granules give large proportions of void space in the die before compression and relatively light weight tablets. It has been shown (3) that as the particle size is reduced, the average tablet weight of batches increases until, for still smaller particles, the average weight tends to decrease. This is probably due at least in part to phenomena connected with bulk density (5–7).

Large granules also give large tablet weight variation. This is presumed to be caused by variation in the proportion of voids, attributable in part to the varying amount of breakage of granules and the removal of the resulting powder by the movement of the feedshoe of the single punch press. Here also, the effect is initially reduced by a decrease in particle size. As the particle size decreases, the weight variation decreases, passes through a minimum, and then increases. It seems that in this case again other factors such as air entrapment and the impairment of the free flow of granulation tend to counteract the basic effect.

It would seem that in general the conditions that lead to a minimum variation in weight also work toward a maximum average weight. The sodium chloride data are in close agreement with that postulate as 324 μ granules give a minimum weight variation and 230 μ granules give a maximum average weight.

It should be noted that the sodium chloride was fractionated into more narrow size ranges than the lactose-starch and contained neither lubricants nor a disintegrant. In the case of the lactose-starch tablets, therefore, the size relationships would deviate from and usually be somewhat smaller than the sizes listed.

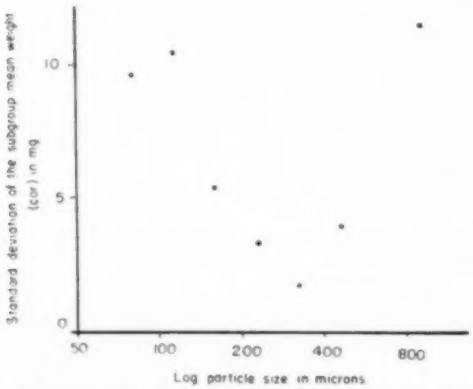

Fig. 3.—Weight variation vs. particle size of sodium chloride tablets.

All batches compressed from fractions of sodium chloride granulation between 920 and 81 μ were within the control limits except for a few samples. Those few subgroups which fell outside of the control limits were, with one exception, the initial or final samples of the batch. The U. S. P. weight variation test was applied to each batch. Batches 1840, 1095, 855, and 545 μ of the lactose-starch granulation, and batches 460, 324, 230, 163, and 115 μ of the sodium chloride granulation were satisfactory.

The determination of the point of minimum weight variation can also be based on the average range ($\bar{R}_{cor}$). For this point substantially the same minima were obtained, the lowest value for sodium chloride falling at 324 μ. For the lactose-starch granulation, the weight variation based on $\bar{R}$ is slightly less for 855 μ than for 545 μ.

Statistical quality control charts were prepared for all batches of lactose-starch and sodium chloride tablets. The examination of the charts helped to guard against accepting values which had been affected by such sources of error as shifts in machine adjustment or the substantial segregation of granulation as discussed by Raff, *et al.* (5). The charts for sizes 230, 324, and 460 μ in Fig. 2, however, indicate that some internal flow occurred in spite of the relative homogeneity of the material. It is believed that this flow was minimized by the more limited duration of hopper vibration caused by the smaller batches. It seems possible that some of the variations exhibited by other sizes were caused by localized stratification or unmixing.

SUMMARY

1. Weight variation was determined for $^{7}/_{16}$-inch tablets made on a single-punch tablet press from lactose-starch granulations and from sodium chloride granulations of relatively uniform particles, using statistical quality control procedures.

2. As the particle size decreased, the tablet weight variation decreased, passed through a minimum, and then increased.

3. The particle size range leading to minimum weight variation was 400–800 μ for lactose-starch, 200–500 μ for sodium chloride.

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Rodent Repellent Studies. IV. Preparation and Properties of Trinitrobenzene-Aryl Amine Complexes*

By JAMES B. DeWITT, ERVIN BELLACK, and JACK F. WELCH

Data are presented on methods of preparation, chemical and physical characteristics, toxicity, and repellency to rodents of complexes of symmetrical trinitrobenzene with various aromatic amines. When applied in suitable carriers or incorporated in plastic films, members of this series of materials were shown to offer significant increases in time required by wild rodents to damage common packaging materials.

IN THE COURSE of studies being conducted by the Fish and Wildlife Service under grants from the Office of the Quartermaster General, it has been found that rodent attacks upon paperboard and other materials may be reduced by application of certain organic compounds (1, 2). The repellent activity of such compounds appears to be associated with specific functional groups or configurations containing nitrogen, halogen, or sulfur. Amines and nitro compounds are among the more active classes, although the presence of both amine and nitro groups on the same carbon chain or ring results in relatively inactive compounds.

Many amines are too volatile to meet the stability requirements for a successful repellent, and it was necessary to convert them to salts or other solid derivatives for testing. Salts with acetic and hydrochloric acids were relatively active, but it was found in a number of cases that amine picrates were much more repellent than either parent compound. Since it had been observed in other series that hydroxy groups had little associated repellency and might decrease repellent activity of other groups, it was decided to replace picric acid with similar compounds which did not possess the hydroxy radical. Trinitrobenzene was found to be highly repellent, while complexes of this compound with various aryl amines were found to be among the most active classes tested.

The preparation and chemical characteristics of a number of amine-trinitrobenzene complexes have been described by other investigators (3–8). Since it was found necessary to make slight procedural modifications in some cases, and since two previously undescribed complexes were prepared in these studies, details of preparation and properties of the series are included in this paper.

* Received April 9, 1953, from the U. S. Department of Interior, Fish and Wildlife Service, Laurel, Md.

EXPERIMENTAL

Preparation of Complexes.—One-thirtieth mole of sym. trinitrobenzene was dissolved in approximately 100 ml. of methanol at room temperature. To this solution was added one-thirtieth mole (or a slight excess) of the amine dissolved in the minimum amount of methanol. The mixed solutions were heated to boiling, cooled, and the crystals collected on a Büchner funnel. Occasionally, crystals began forming during the boiling process, but more frequently the solution had to be concentrated to 50 ml. or less before satisfactory yields could be obtained. Products were recrystallized from methanol with extreme care so as to remove all traces of solvent from the final crystals. The properties of these complexes are given in Table I.

Toxicity.—Finely ground samples were suspended in gum acacia solution and administered by stomach tube to individually caged laboratory rats. Following dosing, the animals were allowed *ad libitum* access to normal food and water. The surviving animals were held for observation for a period of at least five days following the administration of the test material.

Repellency.—The initial determination of repellent activity was made according to the procedure described by Bellack and DeWitt (9). In this procedure, individually caged laboratory rats were given 20 Gm. of a 2% mixture of the test material in ground laboratory chow and 20 Gm. of similar untreated food. Water was furnished *ad libitum* and food consumption determined daily during the four-day assay period. The repellency index (K) was calculated by the equation:

$$K = 100 - 1/100W(8T_1 + 4T_2 + 2T_3 + T_4)(U_1 + U_2 + 2U_3 + 4U_4 + 8X),$$

where W = body weight (in Kg.), $T_1 \ldots T_4$ represent Gm. of treated food consumed on each day of test, $U_1 \ldots U_4$ represent Gm. of untreated food eaten each day, and X represents Gm. of untreated food remaining at the end of the test period.

Further evaluation of the repellent activity was made in barrier tests (10), or in field exposure stations (11). In the barrier tests, the test material was applied in lacquer or other carrier as a surface coat on paperboard panels inserted between specially trained laboratory rats and their food supply. The average time required for penetration of the treated paperboard as compared with that required for penetration of the untreated control panels was taken as a measure of the relative effectiveness of the treatment. In field exposure studies, the test material was applied as a surface coat on sealed paperboard boxes exposed under simulated warehouse conditions to attack by wild Norway and roof

TABLE I.—CHEMICAL AND PHYSICAL PROPERTIES OF TRINITROBENZENE-ARYL AMINE COMPLEXES

Parent Amine	Yield, %	M. P., °C.	Cryst. Form and Color	Solubility in CH_3OH	Literature Reference
Aniline	76	126–127	Orange cryst.	V. sol.	5
o-Chloroaniline	90	135–136	Orange need.	Sol.	3
m-Chloroaniline	80	116–118	Orange need.	V. sol.	3
p-Chloroaniline	90	96–98	Orange need.	Sol.	3
p-Bromoaniline	80	114–116	Orange need.	Sol.	3
o-Toluidine	90	129–130	Orange-red need.	V. sol.	4
m-Toluidine	95	93	Orange-red need.	V. sol.	4
o-Anisidine	90	96–98	Dark red need.	Sol.	3
p-Anisidine	98	80–82	V. dk. red need.	Sol.	3
o-Phenylenediamine	99	170–172	Red-blk. need.	Sol.	8
m-Phenylenediamine	95	162–163	Black cryst.	Sol.	8
p-Phenylenediamine	95	144–145	Black need.	Sol.	8
6-Chloro *o*-toluidine	80	132–134	Orange need.	V. sol.	None
1-Naphthylamine	99	214	Dark red cryst.	Sl. sol.	6
2-Naphthylamine	97	162	V. dk. red cryst.	Sl. sol.	6
Xylidines (mixed)	85	95–105	Dard red need.	V. sol.	None
Tetramethyldiamino-diphenylmethane	98	114	Purple-blk. need.	Sol.	7
Acridine	85	115	Yellow need.	Sol.	3
Allylamine	?	?	Orange cr. (impure)	V. sol.	None
Nicotine	?	?	Brown fl. (impure)	V. sol.	None
Quinaldine	?	?	Brown fl. (impure)	V. sol.	None
Dodecylamine	100	?	Reddish-blk. tar	V. sol.	None
o-Tolylethanolamine	100	?	Dk. red sol. (pasty)	V. sol.	None

rats. In a modification of these tests, two of the complexes were incorporated in vinyl films applied as a loose wrap to cartons exposed to attack by wild rodents.

RESULTS AND DISCUSSION

The general method for preparation of amine-trinitrobenzene complexes was found satisfactory for the majority of aromatic amines tested and yielded crystalline products having definite melting points. The reaction was almost instantaneous, as evidenced by the rapid development of colored solutions. With a few aromatic amines, notably *o*-aminodiphenyl, no reaction occurred and the trinitrobenzene crystallized out of the solution unchanged. Similar results were obtained with heterocyclic amines, such as 2-amino-3-methyl pyridine and 2-vinyl pyridine, while other heterocyclic compounds (e. g., nicotine and quinaldine) formed complexes which were either unstable or contaminated with considerable quantities of unchanged trinitrobenzene. Aliphatic amine complexes could not be prepared by this procedure, but dodecylamine-trinitrobenzene was prepared by direct addition of the amine to dry trinitrobenzene. The reaction was exothermic, and the product was pasty or tarry. Melting points and other physical characteristics of such products could not be determined. Chemical and physical properties of the crystalline complexes prepared in this study are given in Table I.

Data on oral toxicities and repellency indices of these complexes are presented in Tables II and III. The minimum lethal dose for most of the materials was approximately the same as that for the parent amines and was lower than that of free trinitrobenzene. In a few cases, the complex was more toxic than either component, but none appeared sufficiently toxic to preclude its consideration as a protectant for packaging materials or other commodities. However, long-range toxicological tests will be required to determine whether the use of these complexes involves the possibility of contamination of foods or offers hazards to the personnel manufacturing or handling the treated materials.

With the exception of the complex of tetramethyl diamino diphenyl methane, all members of the series gave repellency indices well above the minimum value (85) necessary for consideration as possible repellents. The high indices, coupled with favorable results obtained in barrier tests on three complexes, indicated that the intermediate screening process could be by-passed and the majority of the materials were placed immediately on field exposure trials.

TABLE II.—TOXICITY TO RATS OF ARYL AMINES AND THEIR COMPLEXES WITH TRINITROBENZENE[a]

Parent Amine	Minimum Lethal Dose (Mg./Kg.) Free Amine	TNB Complex
Aniline	900	750
o-Chloroaniline	750	750
m-Chloroaniline	200	250
p-Chloroaniline	250	250
p-Bromoaniline	500	500
o-Phenylenediamine	500	500
m-Phenylenediamine	500	250
p-Phenylenediamine	100	500
o-Toluidine	600	1,000
m-Toluidine	500	500
o-Anisidine	700	750
p-Anisidine	500	500
6-Chloro-*o*-toluidine	700	500
Xylidines (mixed)	Above 700	250
1-Naphthylamine	500	700
2-Naphthylamine	750	250
Tetramethyldiamino-diphenylmethane	...	750

[a] Sample of sym. Trinitrobenzene had M. L. D. of 750 mg./Kg.

TABLE III.—REPELLENCY TO RATS OF AMINE-TRINITROBENZENE COMPLEXES

Complex	Test Procedure: Food Acceptance K Values at Levels of 2%	1%	0.5%	Barriers F[a]	Field Exposure: Carrier	Concentration, Gm./Sq. In.	F[a]
Aniline	100.0	99.5	98.5	1.5	Starch	0.05	10.4
					Vinyl	0.045	8.25[b]
o-Chloroaniline	99.0	95.4	96.4	...	...	...	...
m-Chloroaniline	100.0	98.0	98.6	...	Starch	0.05	2.3
p-Chloroaniline	100.0	100.0	99.4	...	Starch	0.05	2.5
p-Bromoaniline	100.0	88.0	87.0	...	...	...	...
o-Phenylenediamine	99.0	94.0	96.0	...	...	...	...
m-Phenylenediamine	96.0	...	...	5.8	Starch	0.05	3.8
p-Phenylenediamine	96.5	89.5	95.3	...	...	...	...
o-Toluidine	100.0	98.3	99.2	7.3	Starch	0.05	2.0
m-Toluidine	95.5	99.2	99.1	...	Starch	0.05	2.3[b]
o-Anisidine	99.3	100.0	97.1	2.6	Starch	0.05	9.5[b]
					Vinyl	0.025	8.25[b]
p-Anisidine	95.0	90.3	85.9	...	Starch	0.05	3.8[b]
6-Chloro-*o*-toluidine	100.0	99.6	97.9	...	...	...	...
Xylidines (mixed)	96.0	100.0	98.5	...	Starch	0.05	4.6
1-Naphthylamine	100.0	100.0	99.8	3.5	Starch	0.05	2.6
2-Naphthylamine	94.2	100.0	98.1	...	Starch	0.05	3.6
Tetramethyldiamino-diphenylmethane	75.0	...	...	...	...	...	...
Allylamine	100.0	?	?	...	...	...	...
Nicotine	100.0	?	?	...	...	...	...
Quinaldine	96.0	?	?	...	...	...	...
Dodecylamine	94.0	?	?	...	...	...	...
o-Tolylethanolamine	95.7	?	?	...	...	...	...

[a] F = time required for penetration of treated material/time required for penetration of untreated control.
[b] Some treated cartons undamaged at end of test period.

For the purposes of these field tests, the complexes were applied in starch paste or other carrier to the surfaces of solid fiberboard (V_2S) cartons at a concentration of 0.05 Gm. per square inch. With all complexes tested, the treated cartons withstood rodent attack for periods appreciably longer than those required for penetration of similar untreated cartons exposed simultaneously. Two of the more effective complexes, aniline-trinitrobenzene and *o*-anisidine-trinitrobenzene, were incorporated in vinyl films (2) and applied as loose wraps. In these cases, increases in protection were more marked than with other methods of application.

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Infrared Analysis of Active Ingredients in Ointments*

II. Pilocarpine Hydrochloride and Phenacaine Hydrochloride

By WILLIAM H. WASHBURN

An infrared method for the analysis of pilocarpine hydrochloride and phenacaine hydrochloride in ointments is described. Pilocarpine hydrochloride in the amount of 2 per cent may be determined in a 1-gram sample with an accuracy of ±4 per cent. Phenacaine hydrochloride in the amount of 1% may be determined in a 1-gram sample with an equal accuracy.

IN A PREVIOUS paper (1) infrared methods were described for atropine sulfate and scopolamine hydrobromide in ointments. The infrared method is an improvement over the usual titrimetric methods for this type of compound in that it is specific and practical for the assays of small amounts of active ingredients. The method has now been applied to the determination of pilocarpine hydrochloride and phenacaine hydrochloride in ointments.

EXPERIMENTAL

Spectra of both pilocarpine base and phenacaine base were recorded. Pilocarpine (Fig. 1), a colorless oily material, was prepared by pressing a drop of material between two rock salt plates. Phenacaine, the spectrum of which is shown in Fig. 2, was prepared by evaporating ½ cc. of a 1% carbon tetrachloride solution on a rock salt plate. Per cent transmittance curves were constructed from the single beam records (2).

In ointments, water-soluble salts of both pilocarpine and phenacaine are employed. This necessitates conversion of the salt to free bases in order that a suitable nonpolar solvent may be employed for quantitative infrared work.

In the case of pilocarpine, chloroform was selected as the solvent, and for phenacaine, carbon tetrachloride was found to be the solvent of choice. The absorption band at 9.01 μ was selected for the quantitative determination of pilocarpine. For phenacaine, the band at 9.52 μ was chosen. The ointment base in these preparations consists of lanolin, petrolatum, and mineral oil.

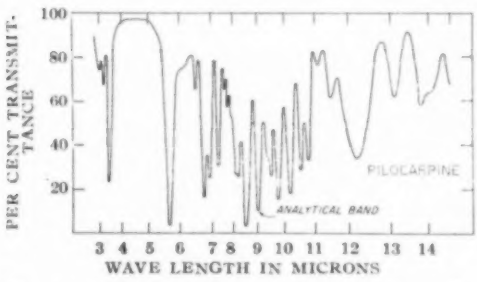

Fig. 1.—Infrared absorption spectra of pilocarpine.

Working curves were constructed as follows: several portions of pure pilocarpine hydrochloride were dissolved in water and made slightly alkaline by the addition of 15% ammonium hydroxide. The pilocarpine base was then extracted with four 10-cc. portions of chloroform, and the chloroform extracts were evaporated to dryness. The residues were diluted in chloroform to known concentrations ranging from 3–8 mg./cc., and using a cell of 0.5 mm. thickness, absorbancies were noted at 9.01 μ. Absorbancies were then plotted against concentration.

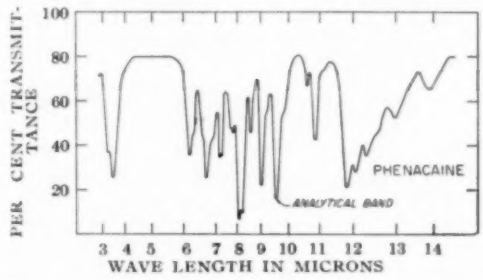

Fig. 2.—Infrared absorption spectrum of phenacaine.

In the construction of a working curve for phenacaine hydrochloride, the same procedure was used with the following exceptions: The aqueous phase was made alkaline with 1 *N* sodium hydroxide, the residues were taken up in carbon tetrachloride, and absorbancy measurements were made at 9.52 μ. Beer's law is followed closely in both cases.

Throughout this study the measurements were made with a Perkin-Elmer model 12C infrared recording spectrometer equipped with sodium chloride optics and a three-speed slit drive.

Quantitative Infrared Determination of Pilocarpine Hydrochloride 2% in Ointment.—Transfer about 1 Gm. of ointment (accurately weighed) to a suitable flask. Dissolve in 15 cc. of ether and transfer to a separatory funnel. Rinse flask with two 10-cc. portions of ether and add to the separatory funnel. Extract with five 10-cc. portions of water, rinsing each portion first through the original flask and collect the aqueous phase in a second separatory funnel. Render the aqueous phase alkaline with 15% ammonium hydroxide, and extract with four 10-cc. portions of chloroform. With the aid of a stream of nitrogen evaporate the chloroform to dryness in a 50-cc. flask.

Dissolve the residue in 5 cc. of 2% hydrochloric acid and heat on a steam bath. Transfer the solution to a separatory funnel. Rinse the original flask with two 10-cc. portions of 1% hydrochloric

* Received March 25, 1953, from the Control Department, Abbott Laboratories, North Chicago, Illinois.

acid, and add the rinsings to the separatory funnel. Wash the aqueous solution with four 10-cc. portions of ether. Render the aqueous phase alkaline with 15% ammonium hydroxide, and extract with four 10-cc. portions of chloroform. Filter the combined extracts through a chloroform soaked pledget of cotton into a small flask, evaporate to dryness under a stream of nitrogen, and dry for one hour in a vacuum desiccator.

Dissolve the residue in 2 cc. of chloroform and using a sealed cell of 0.5 mm. thickness, determine the absorbancy at 9.01 μ. Refer the observed absorbancy to the working curve and determine the concentration of the solution. Confirm the identity of pilocarpine by scanning the spectrum from 8–10 μ

Table I lists a series of determinations on ointments of known strength and on commercial preparations.

TABLE I.—RESULTS OF ANALYSES OF PILOCARPINE HYDROCHLORIDE OINTMENT 2%

Laboratory Preparations			
Sample No.	Theoretical, Mg.	Found, Mg.	Recovery, %
1	10.2	10.25	100.5
2	10.0	9.99	99.9
3	10.0	9.78	97.8
4	10.0	10.43	104.3
5	9.75	9.8	100.5
6	9.75	10.15	104.1
7	9.8	10.1	103.1

Commercial Ointments			
Sample No.	Theoretical, Mg.	Found, Mg.	Label Claim, %
1	10.07	10.35	102.8
	10.41	10.60	101.9
2	10.02	8.85	88.3
	10.18	8.75	86.0
3	10.15	9.5	93.6
	9.92	8.9	89.7
4	10.37	10.2	98.4
	10.46	10.45	99.9
5	10.36	10.75	103.8
	10.36	10.30	99.4

Quantitative Determination of Phenacaine Hydrochloride 1% in Ointment.—Transfer about 0.5 Gm. of ointment (accurately weighed) to a suitable flask. Dissolve in 15 cc. of ether and transfer to a separatory funnel. Rinse the flask with two 10-cc. portions of ether and add to the separatory funnel. Extract with five 10-cc. portions of water, rinsing each portion first through the original flask, and collect the aqueous phase in a second separatory funnel. Render the aqueous phase alkaline with 5 cc. of 1 *N* sodium hydroxide, and extract with four 10-cc. portions of chloroform. Collect the chloroform phase in a second separatory funnel. Wash the chloroform extracts with one 10-cc. portion of 0.05 *N* sodium hydroxide and drain the chloroform to a small flask. Evaporate to dryness under a stream of nitrogen and dry under vacuum for one hour. (Additional purification of phenacaine is not necessary as it is in the case of pilocarpine, because the absorption of any ointment base carried over is negligible at 9.52 μ.)

Dissolve the residue in 2 cc. of carbon tetrachloride. (If the residue does not dissolve entirely within three minutes, apply heat gently to the tightly stoppered flask.) When solution has been effected, immediately determine the absorbancy of the sample at 9.52 μ in a sealed cell of about 0.5 mm. thickness. Refer absorbancy to the working curve and determine the concentration of the solution. The identity of phenacaine may be confirmed by scanning the spectrum from 9–10 μ.

Table II lists a series of determinations on ointments of known strength and on commercial preparations.

TABLE II.—RESULTS OF ANALYSES OF PHENACAINE HYDROCHLORIDE OINTMENT 1%

Laboratory Preparations			
Sample No.	Theoretical, Mg.	Found, Mg.	Recovery, %
1	10.0	10.08	100.8
2	10.9	10.84	99.4
3	10.9	11.3	103.7
4	10.7	10.9	101.9
5	10.1	9.8	97.0
6	10.0	9.6	96.0
7	10.7	10.9	101.9

Commercial Ointments			
Sample No.	Theoretical, Mg.	Found, Mg.	Label Claim, %
1	10.22	10.06	98.4
	10.12	9.7	95.8
2	10.06	9.89	98.3
	10.39	10.06	96.8
3	10.22	9.23	90.3
	10.17	9.16	90.1
4	10.4	11.55	111.1
	10.72	11.59	108.1
5	10.16	9.94	97.8
	10.30	10.13	98.3

SUMMARY AND CONCLUSIONS

1. An infrared method has been developed for the determination of small amounts of pilocarpine hydrochloride and phenacaine hydrochloride in ointments.

2. Accuracy of the method for each determination is in the range of ±4%.

3. The method is specific in that both pilocarpine and phenacaine may be identified by their characteristic infrared absorption.

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Blood and Bone Marrow Effects of Feeding Zinc Sulfate to Rats and Dogs*

By ERNEST C. HAGAN, JACK L. RADOMSKI, and ARTHUR A. NELSON

Because of its emetic properties zinc sulfate has been proposed for inclusion in barbiturate preparations to prevent deaths due to overdosage of these hypnotics. Since this would lead to chronic ingestion of zinc sulfate in persons receiving prolonged barbiturate medication, we have studied the chronic toxicity of this emetic. Rats were fed zinc sulfate ($ZnSO_4.7H_2O$) incorporated in ground commercial Purina Laboratory Chow at 1,000, 500, 100, and 0 parts per million for twenty-one months. Four of six dogs all maintained on Purina Chow received zinc sulfate by capsule for seventy weeks. The initial daily dose for dogs was 200 mg./Kg. Emesis forced a change of dosage to 100 mg./Kg. at seven weeks, and again in three of four dogs at thirty-two weeks to 50 mg./Kg. The other dog was changed because of emesis to 50 mg./Kg. after three weeks on the 100 mg/Kg. level. Effects observed in rats were microcytosis, coupled with polychromasia in some cases, and hyperchromia in others, and a hypochromic anemia in dogs. The blood effect noted in rats was reversed despite continued zinc feeding. The average myeloid-erythroid ratio of rat bone marrow smears taken at the termination of the experiment ranged from 1.16 to 1.35 for the various groups of zinc animals and read 2.14 for the untreated controls. Dog bone marrow also studied at the termination of that experiment was slightly hyperplastic. Although no differential marrow count was made, the myeloid-erythroid ratio appeared unchanged.

ZINC SULFATE is used medicinally as an emetic, and this has led to the suggestion by Miskimon and Miskimon (1) that it be added in small amounts to barbiturate preparations so as to cause vomiting when these drugs are taken in overdosage either accidentally or with suicidal intent. A corollary of the use of this drug combination would be the ingestion and retention of zinc by the person using reasonable daily amounts of barbiturates. When confronted with this latter possibility we felt it desirable to study the chronic effects of feeding zinc sulfate to laboratory animals.

Smith and Larson (2) fed zinc carbonate to rats for five weeks at a level providing 1.0 per cent zinc and observed a microcytic hypochromic anemia together with retardation of growth. The hemoglobin deficiency failed to develop when a mixture of iron, copper, and cobalt were fed in the anemia-producing diet, while dietary liver produced significant growth response in the retarded animals. Sadasivan (3) fed zinc oxide to rats at 0.5 and 1.0 per cent for fifteen days, and found it to exert a lipotropic effect. Further findings were failure to grow normally and retardation in development and mineralization of bone. At the 1.0 per cent level of zinc oxide feeding Sadasivan (4) observed rats to excrete increased amounts of nitrogen through urine and feces. At 0.5 per cent dietary zinc oxide urinary excretion of phosphorus and sulfur decreased, while urinary excretion of uric acid and creatinine increased. Fecal excretion of phosphorus and sulfur increased. Sutton and Nelson (5) fed rats zinc carbonate at levels corresponding to 0.10, 0.50, and 1.0 per cent zinc. The 1.0 per cent level was found overwhelmingly toxic while impairment of reproduction and decrease in hemoglobin concentration occurred at 0.5 per cent. Hegsted and co-workers (6) have reviewed the literature pertaining to zinc. Drinker, *et al.* (7), reported negative results for production of anemia after administering zinc oxide to dogs and cats. Doses fed to dogs were 1,000 and 500 milligrams per animal for feeding periods of fifteen, and three to nineteen weeks, respectively. Doses ranging from 600–1,000 milligrams were poorly tolerated by cats, and the maximum time such levels were fed was twenty-one weeks. The longest period of feeding at any level was that fed to 1 cat and measured 175 milligrams for eight weeks, then 354 milligrams for forty-five weeks. Drinker, *et al.* (8), fed zinc to rats in daily doses of 0.5 to 34.4 milligrams for from thirty-five to fifty-three weeks. Zinc was fed as the oxide, acetate, citrate, and malate. Blood studies made prior to autopsy failed to reveal abnormalities.

EXPERIMENTAL RESULTS

A.—Four groups of eight Osborne Mendel weanling rats (4 males and 4 females) were fed *ad libitum* a basal diet of ground commercial Purina Chow containing 1,000, 500, 100, and 0 p. p. m. of zinc sulfate ($ZnSO_4.7H_2O$). The rats were individually housed in wire mesh cages. Food consumption and animal weight were recorded weekly. Eight complete blood counts were made which were confined to the

* Received March 25, 1953, from the Division of Pharmacology, Food and Drug Administration, Department of Health, Education, and Welfare, Washington, D. C.

first three and last five months of the experiment. A study of bone marrow smears was made at the termination of the experiment.

During the approximately twenty-one-month feeding period, food intake and weights of the rats fed zinc varied within normal limits. Hemoglobin values, red cell, white cell, and differential counts remained normal throughout the experiment. However, inspection of smears showed the occurrence of microcytosis, coupled with polychromasia in some cases and hyperchromia in others. These blood changes occurred at all levels of feeding, and were first observed at the 1,000 p. p. m. level on the resumption of blood studies at the sixteenth month. One month later this change was noted at the 500 and 100 p. p. m. levels of feeding. Despite the continuation of these rats on the zinc-containing diets the red cells subsequently returned to normal size. Counts (500 cells each) of the bone marrow smears taken at autopsy revealed the average myeloid-erythroid ratio (M/E) of rats fed the basic diet to be 2.14 and of the rats fed all levels of zinc sulfate to vary from 1.16 to 1.35. The bone marrow data are given in Table I.

At autopsy, there were no gross pathological changes attributable to zinc, with one possible exception. The kidneys of the male rats on 500 and 1,000 p. p. m. of zinc sulfate were larger and more granular than those of 100 p. p. m. or the controls. Unfortunately, the kidneys were not weighed. The kidneys of the females were only rarely enlarged or granular, and there was no difference between groups. Liver weights, when expressed as percentage of body weight, showed no difference between groups. All 32 rats were to at least some extent examined microscopically. Six animals given 1,000 p. p. m. zinc sulfate and 6 controls, evenly divided by sex, were examined in detail, while for the remaining 20 animals sections were limited to liver, kidney, testis (if male), and in 8 instances bone and bone marrow. Only the kidney and bone marrow showed microscopic changes which could be attributed to zinc. In the bone marrow sections the cellularity of the marrow was alike for all groups, and within normal limits, but the M/E ratios appeared altered, as was better shown by the actual counts of smears. The kidneys of the males, both control and treated, commonly showed at least a slight degree of the spontaneous nephritis (sometimes called nephrosis) which is frequently seen in older rats. The condition is described and illustrated by, among others, Saxton and Kimball (9). Among all the females there was but one in which this condition was present in as much as a moderate degree. Among the males, however, there were 6 with the condition in moderate degree, and 5 with it in severe degree. All the latter 5 animals were in the 500 and 1,000 p. p. m. groups, suggesting, if not proving, that zinc increased the severity of the normally occurring nephritis or nephrosis in males.

B.—The toxicity of zinc sulfate to dogs was also determined. A litter of 6 Dalmatian puppies, ten weeks old, was selected for the experiment. Four of the 6 dogs were given zinc sulfate by capsule at a daily level of 200 mg./Kg. to start. Complete blood counts were taken every two weeks at the start of the experiment and at longer intervals thereafter. No counts were taken from the eighth to the thirtieth week of the experiment because of the absence of a technician. After seven weeks of zinc sulfate administration at this level most of the dogs began to vomit consistently after their doses, and the daily dosage level was reduced to 100 mg./Kg. After three weeks on this dose, dog No. 5 began to vomit again, and its dose was reduced to 50 mg./Kg. daily. After thirty-two weeks on 100 mg./Kg. daily, the other 3 experimental dogs began to vomit fairly consistently after dosage and their daily dose was likewise reduced to 50 mg./Kg. At this time dog No. 6 was losing weight and appeared to be in bad physical condition, though all other dogs showed normal appetite and growth throughout experimentation. This dog was sacrificed *in extremis* one week later. After seventy

TABLE I.—BONE MARROW COUNTS—ZINC SULFATE

1,000 P. P. M.				500 P. P. M.			
Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio	Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio
5605	49	44	1.11	5613	50	38	1.32
5606	48	41	1.17	5614	51	41	1.24
5607	51	40	1.27	5615	48	45	1.07
5608	49	43	1.14	5616	49	36	1.33
5609	55	45	1.22	5617	50	44	1.14
5610	44	46	0.96	5618	47	40	1.17
5611	50	48	1.04	5619	49	47	1.04
5612	58	40	1.42	5620	46	46	1.00
Av.	50.5	43.0	1.17	Av.	48.7	42.1	1.16
100 P. P. M.				**Control**			
Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio	Animal Number	Total Myeloid, %	Total Erythroid, %	M/E Ratio
5621	52	40	1.30	5629	60	28	2.14
5622	60	33	1.52	5631	58	29	2.00
5623	57	40	1.43	5632	63	29	2.17
5624	53	40	1.33	5633	67	21	3.19
5625	54	37	1.73	5634	64	28	2.28
5626	45	46	1.00	5635	64	34	1.88
5627	49	45	1.09	5636	54	38	1.42
5628	53	37	1.43				
Av.	52.8	39.8	1.35[a]	Av.	61.4	29.6	2.14

[a] Probability <0.01.

weeks the experiment was terminated and all surviving dogs were sacrificed.

Blood studies of the dogs receiving prolonged daily doses of zinc sulfate showed that although all 6 dogs had, because of their young age, low hemoglobin values at the start of the experiment, they soon attained normal adult hemoglobin values. However, the 4 experimental dogs all eventually developed hypochromic anemia, in that the hemoglobin values declined while the red cell counts remained at normal levels (Table II). No significant effects on the white blood count, total or differential, were noted. Histopathological examination of from 20 to 25 tissues of each of these dogs revealed the only change attributable to zinc to be in the bone marrows, which were uniformly slightly hyperplastic as compared with the controls.

TABLE II.—THE EFFECT ON THE BLOOD HEMOGLOBIN OF ZINC SULFATE ADMINISTRATION TO DOGS

	Hemoglobin—Gm. per 100 Cc. of Blood					
	Untreated		Treated			
Weeks	Dog No. 1	Dog No. 2	Dog No. 3	Dog No. 4	Dog No. 5	Dog No. 6
0	10.0	12.0	10.0	10.0	8.5	10.5
3	10.5	11.0	11.5	11.5	10.5	11.5
5	12.2	12.0	14.0	13.0	13.0	11.9
7	13.0	12.0	11.0	13.0	11.0	11.5
31	14.0	14.0	12.0	13.5	12.8	12.5
29	13.5	15.0	13.5	13.5	11.2	9.0[a]
53	14.0	15.0	12.5	14.0	13.0	...
61	15.0	14.0	10.8	11.0	12.0	...
70	14.0	14.0	10.5	10.0	9.0	...

[a] This value was taken before the dog was sacrificed after 33 weeks on experiment.

Marrow counts were not done on the dogs, but by inspection of sections and smears the M/E ratio did not appear altered.

C.—It was believed desirable to attempt to confirm the results reported by the Miskimons that zinc sulfate is effective in preventing poisoning by fatal doses of pentobarbital. We determined that 8 Gm. of zinc sulfate consistently produced vomiting in twenty to forty-five minutes in dogs concomitantly receiving 450 Gm. of horse meat. Then, to a group of 4 dogs, 8 Gm. of zinc sulfate and 4 Gm. of pentobarbital were administered along with 450 Gm. of horsemeat. This experiment was repeated, omitting the horsemeat. Whether or not the administration of the zinc sulfate-pentobarbital combination was accompanied by the feeding of horsemeat, all 4 of the dogs exhibited emesis in twenty to forty-five minutes. Only 1 of the 4 exhibited sedation, this lasting in both experiments from fourteen to eighteen hours. The other 3 dogs showed no effects from the pentobarbital administration. However, when the dogs were given the same dose of pentobarbital alone, all 4 succumbed within thirty hours. These results indicate that zinc sulfate is effective in preventing poisoning by fatal doses of pentobarbital.

DISCUSSION

Because of the relative leveling off of the growth of rats at about the sixteenth week, the amounts of zinc sulfate subsequently ingested measured about 4–6 mg./Kg. daily, at the 100 p. p. m. level. To correlate this and human consumption it is felt that the ingestion by a human being of a single overdose of 10 or even fewer therapeutic doses (usually 100 milligrams each) of a barbiturate, could easily have serious consequences. The human emetic dose of zinc sulfate is commonly accepted as being from 1 to 2 Gm. The Miskimons found emesis to occur in dogs with 0.3- to 0.5-Gm. doses of zinc sulfate given in single capsules. An increase in the amount of zinc sulfate was found necessary when the dose was divided into several capsules. These investigators also found that the usual dose of zinc sulfate required for emesis in dogs was considerably increased by the simultaneous administration of pentobarbital. It seems probable therefore that each hypnotic capsule intended for human use might have to contain several hundred milligrams of the emetic. The daily intake of zinc sulfate per kilogram of body weight in humans would then be comparable to that which was found to produce adverse effects in rats. It is felt, therefore, that this emetic should not be taken over prolonged periods in amounts that might be included in reasonable barbiturate dosage.

SUMMARY

Zinc sulfate was fed to weanling rats at 1,000, 500, and 100 p.p.m., and to dogs at 200 mg./Kg./day initially, then 100 mg./Kg., and finally 50 mg./Kg. The dose in dogs had to be reduced because of emetic effects. The effects resulting in the rat were microcytosis coupled in some cases with polychromasia and in others with hyperchromia. No reduction of red cell numbers or increase in hemoglobin values occurred. Hypochromic anemia was observed in the dog. The blood changes observed in the rat returned to normal despite continued zinc feeding. The rat bone marrow showed a myeloid-erythroid ratio average of 1.16 to 1.35 in the various groups of zinc animals in contrast to the value of 2.14 found in the untreated controls, all taken on 500 cell counts. The intake of zinc sulfate at the lowest level of rat feeding was approximately 4–6 mg./Kg. daily for the last seventy-seven weeks of feeding. The dog bone marrows showed slight hyperplasia as compared to the controls, with no change in the myeloid-erythroid ratio apparent on inspection.

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1954 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1954 Chilean Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1954 Award, nominations must be received on or before January 1, 1954.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; Paul Block, Louis Gershenfeld, Harvey B. Haag, Lloyd M. Parks, Heber W. Youngken, and Charles O. Wilson.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its sixth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was chosen as the recipient of the fifth award which was conferred during the 1953 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

Book Notices

BOOKS RECEIVED

The Use of Drugs—A Textbook of Pharmacology and Therapeutics for Nurses. By WALTER MODELL, M.D., and DORIS J. PLACE, R.N. Springer Publishing Co., Inc., New York, 1953. 468 pp. 15 × 22 cm. Price $4.50.

Major Metabolic Fuels. Brookhaven Symposia in Biology No. 5, September, 1952. Brookhaven National Laboratory, Upton, N. Y. (Associated Universities, Inc., under contract with the U. S. Atomic Energy Commission.) 234 pp. 18 x 25.5 cm. Paper bound. (Copies can be obtained from the Office of Technical Services, Department of Commerce, Washington 25, D. C.)

Color in Business, Science, and Industry. By DEAN B. BUDD. John Wiley & Sons, Inc., New York, 1952. 401 pp. Illustrated. 15.5 x 24 cm. Price $6.50.

The Pharm-Assist Manual. (Formerly Gray's Pharmaceutical Quiz Compend.) By A. E. SLESSNER. The C. V. Mosby Co., St. Louis, 1953. 167 pp. 13 x 20 cm. Price $3.50.

Farwelliana—An Account of the Life and Botanical Work of Oliver Atkins Farwell, 1867–1944. By ROGERS MCVAUGH, STANLEY A. CAIN, and DALE J. HAGENAH. Cranbrook Institute of Science, Bulletin, No. 34. Cranbrook Institute of Science, Bloomfield Hills, Mich., 1953. 101 pp. 15.5 x 23 cm. Price $0.75.

The Formulary of the Nebraska State Medical Association. 1st ed. Nebraska State Medical Association, 1315 Sharp Building, Lincoln, Neb., 1953. 90 pp. 13 x 19 cm. Paper bound. Price $1.50.

A Manual for the Organic Chemistry Laboratory. By LEIGH C. ANDERSON and WERNER E. BACHMANN. John Wiley & Sons, Inc., New York, 1953. xvi + 164 pp. 23 x 28 cm. Paper and ring bound. Price $2.75.

Soap Manufacture. Vol. I (in 2 volumes). By J. DAVIDSOHN, E. J. BETTER, and A. DAVIDSOHN. Interscience Publishers, Inc., New York, 1953. xii + 535 pp. 16 x 23.5 cm. Price $12.50.

Organic Syntheses. Vol. 33. CHARLES C. PRICE, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1953. 115 pp. 15.5 x 23.7 cm. Price $3.50.

Studies on Dried BCG Vaccine. The Japan BCG Research Council, Tokyo, Japan, November, 1952. 99 pp. 18.5 x 22 cm. Paper bound. Illustrated.

The Natural History of Infectious Disease. 2nd ed. By SIR MACFARLANE BURNET. Cambridge University Press, New York, 1953. 356 pp. 14.5 x 22.5 cm. $4.50.

The author presents the subject from the ecological point of view, describing the interactions between the organisms which cause disease and the host, which must either combat the infection or die. This second edition (the first edition, 1941, bore the title "Biological Aspects of Infectious Disease") contains a new chapter (11 pages) on "The Antibacterial Drugs." The author stresses the dangers of mass chemoprophylaxis, particularly regarding the evolution of resistant varients of pathogens.

The author's fine style and clarity of expression make the text interesting. There is no documentation, but an index is appended.

Ion Exchangers in Analytical Chemistry. By OLOF SAMUELSON. John Wiley & Sons, Inc., New York, 1953. Illustrated. 291 pp. 15.5 x 23.5 cm. Price $6.50.

This well-written and interesting volume presents the text matter in three main divisions. The "General" section discusses principles and properties of ion exchange equilibria, kinetics, and resins. The "Practical" section covers the techniques of ion exchange separations for analysis and chromatography. The major section, "Applications," covers specific methods for inorganic, organic, and biochemical analysis.

The application of ion exchangers for analytical separations is based upon two principles: (*a*) Separation of exchangeable ions from nonexchangeable ions and nonelectrolytes (ion change method). (*b*) Separation of exchangeable ions from each other due to differences in exchangeability (ion exchange chromatography). The first is treated more completely in this text. Each chapter is followed by a bibliography, and author and subject indexes are appended.

New and Nonofficial Remedies 1953. Issued under the direction and supervision of the Council on Pharmacy and Chemistry, American Medical Association. J. B. Lippincott Co., Philadelphia, 1953. xlviii + 623 pp. 13.5 x 19.5 cm. Price $2.65.

The 1953 edition of this very useful and valuable book has a "new look"; it is thinner and the words "Accepted by the Council on Pharmacy and Chemistry" appear on the cover. The book is

thinner because that portion of the text that has appeared as Section *B* will this year appear as a separate volume entitled, "Tests and Standards for New and Nonofficial Remedies."

The decision to publish Sections *A* and *B* separately was made after a survey indicated that few users found it necessary to utilize both sections to meet their particular needs. This probably means that few physicians utilized both sections. Pharmaceutical chemists would probably prefer the combined volume.

The 1953 edition includes general information about the aims and membership of the Council on Pharmacy and Chemistry, its rules for acceptance of articles, and its general decisions. A bibliography of unaccepted products and an index to distributors are also included.

Dr. Robert T. Stormont, Secretary of the Council, repeats the information that the Council does not consider new drugs for inclusion in *N. N. R.* automatically on their release for commercial distribution. The submission of articles for consideration by the Council depends entirely upon the voluntary initiative of the various pharmaceutical firms.

Tests and Standards for New and Nonofficial Remedies, 1953.—Containing Tests and Standards for Nonofficial Drugs and Dosage Forms Which Stand Accepted by the Council on Pharmacy and Chemistry of the American Medical Association on January 1, 1953. Issued by the Chemical Laboratory, American Medical Association. J. B. Lippincott Co., Philadelphia. xi + 325 pp. 13 x 20 cm.

This year, for the first time, New and Nonofficial Remedies has been divided into two parts, resulting in the complete separation of tests and standards from the therapeutic information.

The book contains definitions, structural formulas, physical descriptions, tests for identity and purity, methods of assay for basic drugs and for active ingredients of dosage forms, and tolerance limits of drugs accepted by the Council on Pharmacy and Chemistry for which official standards are not available. In some monographs more than one method of assay is given for the determination of a characteristic group in order to present an alternative method for one requiring special equipment that might not be available to all laboratories.

When the Council on Pharmacy and Chemistry of the American Medical Association is requested to consider a drug for acceptance, the manufacturer, among other data, is required to furnish a considerable amount of chemical information relative to his product. In addition to general information such as the chemical name, empirical and structural formulas, molecular weights, and references to the chemical literature showing proof of structure, certain other criteria are also required. Among these criteria are data on physical properties, identity tests, tests for the detection of impurities, assay procedures, and tolerances permitted in the finished product. Information of this nature is also required by the Food and Drug Administration when a manufacturer files a new drug application. The specifications in New and Nonofficial Remedies are not necessarily identical in every respect with those furnished by the manufacturer since they may differ in minor details. The publication of the specifications in this book, therefore, serves a useful purpose by making them a matter of public record. In addition, methods applied to one drug may often be adapted to other drugs of similar nature, and having these data available may save much time and effort in developing specifications for newly developed drugs. Many present-day official standards have been adapted from specifications which first appeared in N. N. R., and from this standpoint the N. N. R. plays an important role, often not recognized, in the development of official standards for important drugs ultimately appearing in the U. S. P. and the N. F.

Hanna's Handbook of Agricultural Chemicals. By LESTER W. HANNA. Published by Lester W. Hanna, Forest Grove, Oregon. 1952. 209 pp. 13 x 22 cm. Price $3.25.

This handbook, while designed especially for use by the farmer, rancher, orchardist, live stock producer, dairyman, and forester, should also be a welcome addition to the library of every pharmacist who distributes agricultural chemicals to the ultimate user. It will serve as an excellent reference book for information on the various common chemical names and numerical designations of the many and varied agricultural chemicals, so widely used for so many different purposes. The book lists 96 fertilizers, 50 fumigants, 110 fungicides, 53 weed killers, 182 insecticides, 11 rat killers, and 13 miscellaneous materials. The book, however, is more than a mere catalog of names. Information is given concerning the use of all chemicals listed and in many cases warnings concerning their handling.

Another important feature relates to information on the toxicity of many of the compounds covered, not only to humans, but also to farm animals and crops. Where the information is available, the inherent dangers of poisonous residues in food crops to which chemicals have been applied are stressed.

The *Handbook* is bound in a durable, flexible fabricoid cover and the printing is adequate. It is perhaps unfortunate that the author did not include the sources of many of the agricultural chemicals listed in the *Handbook*, because suppliers for many of them are difficult to locate.

Chemistry of Carbon Compounds. Vol. II, Part A. Alicyclic Compounds. E. H. RODD, Editor. Elsevier Publishing Co., Houston, 1953. xx + 487 pp. 16.5 x 23 cm. Price $12.50. (Subscription price $11.25).

Parts *A* and *B* of Volume I of this excellent series were reviewed [THIS JOURNAL, **41**, 169 and 629 (1952)] soon after their publication. As was mentioned in the previous reviews, the object of this new work by a team of distinguished chemists is to fill the need for an up-to-date systematic book, intermediate in size between the encyclopedias such as *Elsevier* and *Beilstein* and the shorter textbooks such as those by Karrer and by Fieser. Its aim is to serve as a successor to Richter's *Organic Chemistry*, and it appears to be a very worthy successor to that important work. *Chemistry of Carbon Compounds* should adequately fill the general needs of practicing organic chemists, including those whose main interests lie in the development of synthetic organic medicinal chemicals.

In Part *A* of the second volume, the chemistry of the nonaromatic carbocyclic compounds is presented in simple lines. The opening chapter discusses theoretical aspects of the structure and stability of saturated carbon rings, after which eight chapters are devoted to the chemistry of the different types of alicyclic compounds, from those containing single rings of all sizes to the more complex spiro, fused ring, and bridged ring compounds. Thus, a good foundation is laid for the understanding of the chemistry of complex chemicals of natural origin, such as the terpenoid and steroid compounds. The group of polyene compounds, known as "carotenoids," has been covered in a separate chapter. Many other natural products are discussed in this volume as members of one of the chemical types to which they structurally belong, as in the chapter dealing with cyclopentane derivatives, where the pyrethrins and the acids related to chaulmoogric acid are included.

As in Volume I, the book is thoroughly documented throughout and is provided with an excellent index. Three additional volumes are planned to complete the series. In Volume III, the aromatic compounds will be covered, Volume IV will be devoted to heterocyclic compounds, and Volume V will include miscellaneous groups not covered in the preceding volumes, and a general index to all five volumes.

Organic Analysis, Vol. I. Editorial Board: JOHN MITCHELL, JR., I. M. KOLTHOFF, E. S. PROSKAUER, A. WEISSBERGER. Interscience Publishers, Inc., New York, 1953. 473 pp. 16 x 23.5 cm. Illustrated. Price $8.50.

Organic nonelemental analysis is of great importance to industrial organic and analytical chemists. Unfortunately quantitative analysis as taught in most universities deals largely with inorganic systems which may or may not include elemental analysis of organic compounds. Most advanced courses stress physical, physicochemical, and instrumental methods of analysis. Thus, the student, except in some colleges of pharmacy, may not become acquainted with the field of organic quantitative nonelemental analysis.

The series *Organic Analysis* is designed to fill this gap in the education of the analytical and organic chemist, and to provide the practicing chemist with a reliable survey of important organic analytical methods. In the first few volumes of the series it is planned to emphasize functional group analysis. Thus, in Volume I the determination of hydroxyl groups, alkoxyl groups, the alpha epoxy group organometallic compounds for determination of active hydrogen, diazomethane for determination of active hydrogen, carbonyl compounds, acetals, and organic sulfur groups are covered in the first eight chapters. The ninth chapter is devoted to the spectroscopic functional group analysis in the petroleum industry.

In subsequent volumes, the analysis of mixtures of similar and dissimilar compounds by procedures such as chromatography, distillation, and countercurrent distribution will be discussed. Judging from Volume I, this series should appeal strongly to practicing analytical and organic chemists, both in academic and industrial positions. The book appears to fill a need that has long existed and the publishers are to be congratulated upon their foresight in attempting to fill an important gap in this area of analytical chemistry.

Pharmacology and Therapeutics in Nursing. 2nd ed. By MARION S. DOOLEY and JOSEPHINE RAPPAPORT. McGraw-Hill Book Co., New York, 1953. xvi + 486 pp. 16.5 x 24 cm. Price $4.50.

This is a relatively elementary textbook designed for nurses. The treatment of the subject of pharmacology in this textbook, however, when compared with earlier textbooks with a similar aim, reflects the advances in the academic training of nurses during recent years.

The book is designed in such a way that pharmacists might find it a useful reference book for use in refreshing their memories and obtaining information on the pharmacology of recently developed drugs such as the antibiotics and the antihistamines.

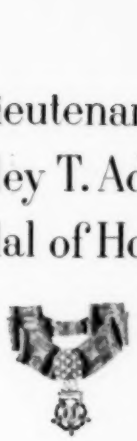
The printing and binding are of good quality and the authenticity of the information presented is assured by the fact that the major author is Dr. Marion Sylvester Dooley, of the State University of New York, College of Medicine, Syracuse, N. Y.

Standard Methods of Clinical Chemistry. Vol. I. (Published by the American Association of Clinical Chemists.) Academic Press, Inc., New York, 1953. xii + 142 pp. 16 x 24 cm. Illustrated. Price $4.50.

This first volume of a series represents the beginning of a project which will fill a need that has long existed. One of the objectives of the American Association of Clinical Chemists involves standardization and codification of clinical laboratory methods in the interest of the public welfare. In order to accomplish this objective it was decided to summarize the methods of clinical chemistry in a form similar to that which has been employed so successfully by *Organic Syntheses*. The plan of organization includes the presentation of individual methods, a delineation of the underlying principle, a precise description of the preparation of reagents and the sequence of procedures, and an indication of the range of normal and pathological results. Whenever the analytical principle can be carried out in two or more ways, the selection has been based on the consensus of clinical chemists working daily with the methods in their own laboratories. By this approach the ultimate standardization of both methods and reagents should be accomplished.

The first volume is restricted to the procedures used more or less routinely in the chemical examination of the blood, such as the determination of bilirubin, chloride, cholesterol, total protein, albumin, and globulin, urea nitrogen, uric acid, and many others. An especially notable chapter is devoted to the determination of sodium and potassium by flame photometry. In choosing the methods to be adopted each one was tested carefully in the laboratory of the submitter and was then sent to one or more checkers who retested it in their own laboratories for validity and practicability. Thus, each procedure described was checked several times on a sufficient number of blood samples from control and pathological cases to insure its validity.

Lieutenant Stanley T. Adams Medal of Honor



ONE BITING-COLD FEBRUARY NIGHT, Lieutenant Adams was on a bitterly contested hill near Sesim-ni, Korea. Out of the dark earth the silhouettes of some 150 Communist troops rose up against the sky-line. Ordering fixed bayonets, the lieutenant, with only 13 men, leaped up and charged furiously against the overwhelming odds. He was knocked down by a bullet. At least three hand grenades actually bounced off his body before exploding near-by. But when Adams and his squad were through, there were only 50 Communists left on the hill—and they were dead.

"Nobody likes to kill," says Stanley Adams. "Nobody likes war. But today the surest way to invite a war is to be weak. You and I know that twice in the last ten years Americans have let their guard down. And the Philippine and Korean graveyards are filled with men who paid the price for it.

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★ ★ ★

Remember that when you're buying bonds for national defense, you're also building a personal reserve of cash savings. Remember, too, that if you don't save *regularly*, you generally don't save at all. Money you take home usually is money spent. So sign up today in the Payroll Savings Plan where you work, or the Bond-A-Month Plan where you bank. For your country's security, and your own, buy United States Defense Bonds now!

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